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with a focus on gene expression and wound imaging

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The thesis is submitted for the degree of Doctor of Philosophy and includes a literature review, 3 scientific publications and unpublished results

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Accompanying papers

Paper I

Przybylska, D. A., Schmidt, J. G., Vera-Jimenez, N. I., Steinhagen, D., & Nielsen, M. E. (2013). β-glucan enriched bath directly stimulates the wound healing process in common carp (*Cyprinus carpio* L.). Fish & Shellfish Immunology 35 (3), pp. 998-1006.

Paper II

Schmidt, J. G. & Nielsen, M. E. (2013). Expression of immune system-related genes during ontogeny in experimentally wounded common carp (*Cyprinus carpio*) larvae and juveniles. Developmental and Comparative Immunology, *in press*, DOI: 10.1016/j.dci.2013.09.003.

Paper III

Schmidt, J. G., Przybylska, D. A., Andersen, E.W., Ersbøll, B.K. & Nielsen, M. E. Long-term investigation of healing of full-thickness cutaneous excisional wounds in rainbow trout (*Oncorhynchus mykiss*). Article submitted to Fish & Shellfish Immunology.

Sammendrag

Fisk fra opdræt er ekstra udsatte for at få vævsskader fra en række sider såsom bidsår fra andre fisk eller prædatorer, hårdhændet håndtering eller infektion. Sårheling hos zebrafisk er relativt velundersøgt, men mest i forbindelse med regenerering af amputerede finner. Sårheling hos de store, hurtigvoksende fiskearter, der opdrættes i konsumøjemed, er kun undersøgt i meget ringe grad.

Målet med dette PhD-studium var at undersøge visuelle og molekylære ændringer under sårheling af hud og muskel hos karpe (*Cyprinus carpio*) og regnbueørred (*Oncorhynchus mykiss*). Begge arter er vigtige produktionsdyr, men i forskellige dele af verden. Karpen er en varmtvandsart, mens regnbueørreden trives i kolde og tempererede egne. De to arters livsstrategi adskiller sig desuden på flere andre områder, og de er ligeledes relativt fjernt beslægtede, hvilket yderligere giver studierne et komparativt tilsnit.

Vi benyttede os hovedsagligt af en model hvor en cylinder med hud og muskel blev fjernet med en biopsistanse. En større biopsistanse blev brugt til at udtage standardiserede vævsprøver under sårhelingen. Samtidig optog vi løbende digitale billeder af sårene til billedanalyse af sårlukningen. Under sårhelingen tilsatte vi bakterier eller immunmodulerende stoffer såsom β-glukaner.

Sårhelingen var hurtigere hos karpen end hos regnbueørreden. Hos begge arter regenererede huden hurtigt, og selv om det beskadigede område i starten adskilte sig fra intakt hud ved at være mørkere, aftog denne hyperpigmentering langsomt med tiden. Huden, der dækkede såret hos karperne, vedblev med at være svagt mørkere, mens den hos regnbueørreden stort set lignede intakt hud et år efter skaden var påført. Derimod regenererede regnbueørredens skæl ikke i det område, hvor huden var fjernet. Vi undersøgte også sårhelingskapaciteten hos karper helt ned til 7 dage efter befrugtning. Disse unge karpelarver udviste en komplet regenerering af det beskadigede væv i løbet af kort tid, hvorimod denne evne var drastisk nedsat efter metamorfosen.

Vi undersøgte udtrykket af en række gener, der er involveret i forskellige stadier af sårhelingen. En række af disse har ikke tidligere været beskrevet fra fisk.

I de tidlige udviklingsstadier havde karperne et meget begrænset transkriptionsrespons. De helt små karpelarver opregulerede dog udtrykket af metalloproteinasen MMP9 og immunglobulinet IgZ1 som følge af vævsbeskadigelse. Efter metamorfosen udviste karperne ingen

vævsskadeinduceret regulering af disse gener. Voksne karper havde en hurtig og kortvarig inflammatorisk respons, der allerede var aftaget efter 3 dage. Regnbueørrederne havde derimod en langtrukken inflammationsfase, der først aftog efter to uger. Ligeledes var transkriptionen af de gener, der var relateret til vævsgendannelse, sent opreguleret. Til gengæld var de stadig opregulerede på den sidste prøvetagningsdag 100 dage efter såret blev påført.

MacroGard er et β-glukan produkt, der er almindeligt brugt som immunmodulator i fiskeopdræt. Vi tilsatte MacroGard til vandet og viste at det stimulerede karper til en hurtigere sårlukning, mens det ikke havde nogen visuel effekt på sårhelingen hos regnbueørred. MacroGard havde kun lille effekt på udtrykket af de undersøgte gener hos begge arter.

Selvom huden hos begge undersøgte arter havde relativt gode regenereringsegenskaber, var det gendannede muskelvæv fibrøst, og var hårdere end ubeskadiget muskel selv efter et år. Da teksturen er en vigtig sensorisk kvalitetsparameter for fødevarer, vil muskelbeskadigelse have indflydelse på spisekvaliteten selv på lang sigt. Desuden tyder vores resultater på at vævsbeskadigelse ikke udelukkende påvirker selve det beskadigede område, men i en hvis udstrækning har en fibrotisk effekt på hele fisken.

Vores undersøgelser har givet ny viden om udtrykket af en række gener under sårheling hos de to undersøgte arter. I karpens larvestadie var vævsgendannelsen perfekt efter påført skade, mens denne evne aftog efter metamorfosen. Hos voksne karper og regnbueørreder var evnen til at gendanne muskelvæv stærkt nedsat. For fiskeopdrætsindustrien betyder dette at produktionsforholdene ikke blot påvirker fiskens velfærd, men også spisekvaliteten af det endelige produkt.

Summary

Fish produced in aquaculture are subjected to potential tissue damage from a range of sources such as conspecific biting, handling and infection. While wound healing of especially fins has been studied extensively in small model fish species such as the zebrafish, not much is known about wound healing in the larger species produced for consumption.

The aim of this PhD study was to investigate wound healing in carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) and the long-term visual and molecular effect of wounding on skin and muscle. Both species are important production animals in different parts of the world. They have very different life-strategies and habitat use. Carp is a warm-water species, whereas rainbow trout inhabit cold and temperate waters. They are distantly related and thus offer comparative aspects to the studies.

We mostly used a model of full-thickness excisional cutaneous wounds with standardized sampling of the wound edge for gene expression and standardized image capture for analysis of wound closure dynamics. We manipulated wound healing by addition of bacteria or immunomodulators such as β-glucans.

Wounds heal faster in carp than in rainbow trout. We found that the skin of both species heal with a large degree of regeneration, although the initial hyperpigmentation of the affected area persist in adult carp although at much lower levels, whereas rainbow trout skin is only weakly long-term hyperpigmented. On the other hand, rainbow trout scales fail to regenerate over the damage area. We also investigated wound healing in carp as young as 7 days post-fertilization. These carp larvae heal wounds very rapidly and with perfect regeneration, whereas this ability diminishes after metamorphosis.

We investigated the expression of a range of genes involved in different wound healing stages. The expression of several of these genes has not previously been investigated in fish.

Carp larvae and juveniles had a very limited transcriptional response to wound healing. However, larvae upregulated the metalloproteinase MMP9 and the immunoglobulin IgZ1 as a result of wounding, whereas juveniles did not show this response. Adult carp had a short inflammatory response, which was largely resolved by day 3, whereas the inflammation phase was much longer in rainbow trout, and did not resolve until after two weeks post-wounding. The induction of the investigated genes related to production of new tissue was delayed until a

week or more post-wounding, but these were upregulated in wounds at least until 100 days post-wounding.

Subjecting wounded fish to the β-glucan product MacroGard, which is an immunomodulator often used in aquaculture, stimulated wound closure in carp, but not in rainbow trout. MacroGard had only a very minor effect on gene expression in both species.

Muscle healed with a large degree of fibrosis, and as long as one-year post-damage muscle tissue was still significantly tougher than uninjured muscle. Since texture is an important sensory quality parameter for foods a history of muscle damage will affect the quality of the final consumer product. Additionally, our results indicate that there is a small systemic fibrotic effect of damage even in muscle tissue not directly affected.

In conclusion the investigations provided new insights into gene expression in muscle during wound healing in the two investigated species, showed a high regenerative capacity in carp larvae and a very limited regenerative ability of muscle in adult fish. From a production viewpoint it is thus important to avoid injuries to the fish not only out of consideration for fish welfare but also due to implications for the final product quality.

1. Objectives of the PhD study

The aim of this PhD project was to study the progression of wound healing in fish with a methodological focus on gene expression and wound image analysis.

We focused on two distantly related species that are popular production animals over much of the globe: The common carp (*Cyprinus carpio*) and the rainbow trout (*Oncorhynchus mykiss*).

For most of the experiments we used a biopsy punch to inflict standardized full-thickness excisional cutaneous wounds that penetrated into the muscle. The healing of these wounds was followed visually by digital image analysis and at the molecular level by gene expression.

We not only followed the natural progression of wound healing, but also manipulated the wounds in different ways, mostly by exposing the wound to different pathogen-associated molecular patterns (PAMPs), and measured the effect on wound closure kinetics and expression of genes relevant to different stages in wound healing.

In relation to aquaculture the muscle (or fillet) is the most relevant part of the fish on which to investigate the effect of wounding, since this is the part that is mostly consumed. Ultimately the objective was thus to gain more insight into the neglected area of healing of deep cutaneous wounds in fish.

2. General introduction

It is not uncommon for fish in aquaculture to be subject to injury from a variety of sources. Viral and bacterial pathogens can cause internal hemorrhages and parasites disrupt the mucosal surfaces [1-5]. Wounds can also result from predators or from conspecific biting [6, 7], or from abrasion against raceways or contusions from sorting procedures.

Wounding leads to poor welfare on the part of the fish and to downgrading of the fillets due to mostly poor visual appeal to the consumer [8]. However, there has been relatively little research focus on the potential effect of such injuries on the sensory quality of the fillet. Some studies report a detrimental effect on flesh quality by previous bacterial or parasitic infection [9-11], but the effect of sterile mechanical trauma to the muscle has received almost no attention in relation to aquaculture.

The zebrafish (*Danio rerio*) is a popular model in many areas of biology, and also in wound healing. Much of what is known about the molecular basis of fish wound healing comes from this species, although very little attention has been awarded muscle wounds. Most investigations have involved fin amputation, which does not affect muscle. Injured fins and many other tissues heal almost perfectly in zebrafish, which is one of the reasons that fish have earned the reputation of having a good regenerative potential. However, it is starting to emerge that muscle does not have the same regenerative capacity [12]. Moreover, the small size and determinate growth pattern probably does not make zebrafish a very good model for fast growing aquaculture fish species.

We instead turned our focus to rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*), which are both important aquaculture species. However, they are distantly related and differ considerably in their life-strategy (see table 1), and they thus provide comparative aspects to the studies. Throughout most of the experiments we conducted during this PhD study we employed a model of full-thickness cutaneous wounds inflicted with a biopsy punch. We investigated the temporal changes in gene expression and wound size over the course of healing.

	Cyprinus carpio	*Oncorhynchus mykiss*
Muscle	Red, white, pink	Red, white Muscle utilized for energy during sexual maturation
Scales	Few large (varies)	Many
Skin	Club cells Dermis and epidermis separate easily Extensive epidermal vascularization.	No club cells Skin thickens during sexual maturation Well developed stratum compactum
Habitat and life strategy	Warm water species (20-25°C optimum) Freshwater, lakes and slow flowing rivers, often in turbid water. Native to Asia.	Temperate water species (10-15°C optimum). Freshwater (but can adopt an anadromous life-strategy), many habitats, but spawning in fast flowing clear water. Native to North America
Diet	Omnivorous	Carnivorous
Max weight	45-50kg	20-25kg
Aquaculture	Cultured for more than 4000 years. Popular culture species throughout southern Eurasia for consumption as well as the aquarium trade.	Cultured for consumption around the temperate parts of the globe. Most important aquaculture species in Denmark

Table 1. Different characteristics of carp and rainbow trout (partially from www.fishbase.org).

We tried to manipulate wound healing by exposing the open wounds to known immunomodulators such as β-glucan as well as to pathogenic bacteria to investigate if there was a transcriptional response or an effect on visual parameters. β-glucan products – and especially MacroGard – are the most common immunomodulators used in aquaculture. The immuno-modulatory effect of these have often been demonstrated when incorporated into feed or when

cells are stimulated *in vitro*, but the *in vivo* effect of direct exposure to injured tissue has not been studied previously.

In the following literature review I provide an overview of the different wound healing stages and the cells that are involved in them. Much of the basic knowledge comes from mammals, but where available I will draw on piscine or amphibian investigations. A multitude of genes are differentially regulated during the wound healing stages and in different cell types. I have chosen to investigate the expression of a few handfuls of some of the more studied genes, which collectively represent different healing stages and cell types. In the thesis, focus will be on these factors, and the deliberate omission of other factors does not necessarily reflect an inferior role of these in wound healing. The involvement of specific stages, cells and factors are temporally intertwined during the course of wound healing, and it is not possible to explain them all in a meaningful, continuous manner. However, each stage and cell type relevant for wound healing, as well as the chosen genes, have each been awarded separate sections. It is thus possible to consult these sections for additional information when needed.

2.1 Wound healing in vertebrates

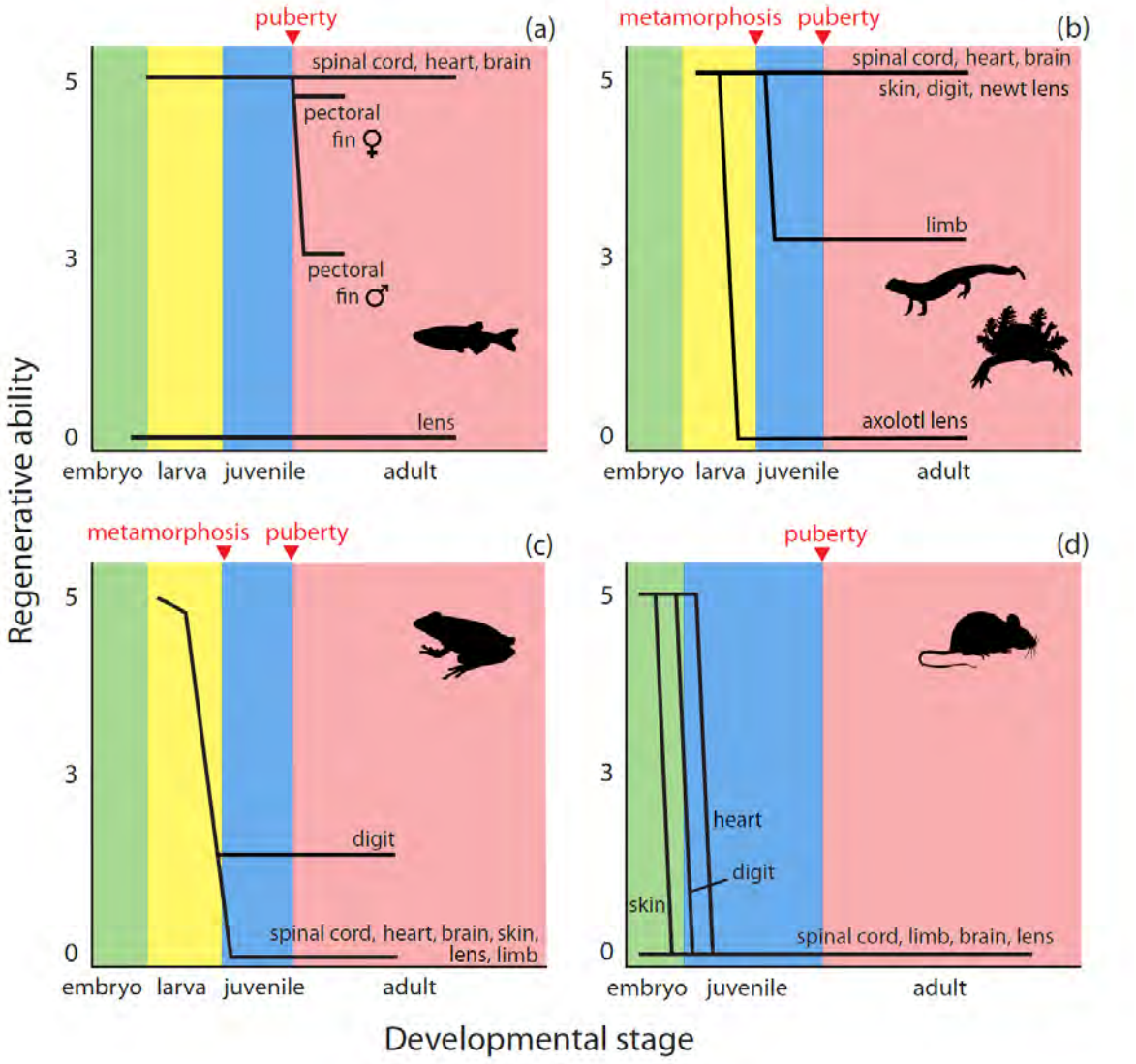


Figure 1. Graphical representation of changes in the regenerative capacity of selected structures during development in zebrafish (a), urodele amphibians and axolotl (b), frogs (c) and mammals (d). Perfect regenerate ability is scored with 5 and 0 represents no regeneration (from [13]).

2.1.1 Mammals

Wound healing can proceed through repair or regeneration. Repair can be more or less fibrotic, but by definition results in a loss of pre-wounding tissue architecture and/or function. On the other hand, regeneration is wound healing with complete gain of function and tissue architecture.

Adult mammalian wound healing is prone to repair, but some tissues such as liver, palatal mucosa and the very tips of digits heal without a scar [14]. Lower vertebrates have a higher regenerative potential (fig. 1), but the overall progression of wound healing is evolutionarily conserved, and consists of a number of partially overlapping stages that varies in magnitude and extent (regeneration from a comparative aspect is reviewed by Brockes and Kumar [15]). Wound healing progression differs with developmental stage, but it may also differ between closely related species or even between races [16]. For example, rodent wound closure occurs mainly by contraction, whereas human wound closure is mostly by re-epithelialisation, and in ponies the inflammation phase is short and robust, whereas in horses it is prolonged but weak [16]. This is well-worth to keep in mind also for fish wound healing when extrapolating from model species such as the zebrafish (*Danio rerio*).

Mammalian embryos and fetuses are known for their ability to rapidly regenerate wounded tissue without scarring [17-19]. Thus comparing adult and fetal wound healing can provide important information that can be used in wound treatment. For example, fetal wounds have a rapid induction of integrin expression, less inflammation with reduced expression of interleukin (IL)-1β, IL-6 and transforming growth factor (TGF)-β1, but with increased expression of TGF-β3, matrix metalloproteinase (MMP)9 and other MMPs [20, 21]. No differentiation of fibroblasts to myofibroblasts is observed during fetal wound healing, and lysyl oxidase (LOX) expression is low [20]. In contrast, dedifferentiation to mesenchymal cells is more often observed in regeneration models than in repair models [14]. These factors, cells and stages will be described in detail later.

One of the central questions in regeneration studies is from where the regenerating tissue derives. There are three basic ways that new, differentiated cells can arise: By dedifferentiation (in which already differentiated cells dedifferentiate, divide and redifferentiate); by transdifferentiation (in which already differentiated cells are reprogrammed to other cell types); and from progenitor cells, that can be either pluripotent or lineage-restricted [22, 23]. This question has been mostly studied in amphibian limb regeneration, where cells do dedifferentiate. However, this is a relatively rare event in vertebrates, and mammalian muscle does not dedifferentiate following injury, but instead regenerate from progenitor satellite cells. However, dedifferentiation can be induced experimentally, *e.g.* by ectopic expression of msx1, which is an important factor for dedifferentiation of myofibers in the urodele and piscine blastema [24-29].

2.1.2 Amphibians

Lower vertebrates heal with less scarring compared to mammals. A special case of regeneration is epimorphic regeneration. *Xenopus* and urodele amphibians such as the axolotl (*Ambystoma mexicanum*) have become important models due to their impressive ability to perfectly regenerate amputated limbs, tails, gills, jaws, lens and heart [14]. Epimorphic regeneration relies on the formation of a blastema consisting of nondifferentiated cells. In recent years it has become increasingly clear that the blastema is not a mass of pluripotent stem cells, but a collection of tissue-specific resident cells with different and restricted potentials [22, 23]. In the case of salamander muscle cells these seem to arise from a combination of stem cells (satellite cells) and dedifferentiating muscle cells [22, 30-32], whereas *Xenopus* muscle cells do not dedifferentiate [33]. The blastema is formed after epithelial coverage of the wound, which is called the apical ectodermal cap and which secretes factors that promote proliferation and keep blastema cells undifferentiated [14]. Although epimorphic regeneration has been mostly studied in association with appendages, *Xenopus* trunk skin wounds seem to regenerate by a similar mechanism [34]. Reports of blastema formation is rare in higher vertebrates, but is reported to occur during antler regeneration in deer [35], and interestingly blastema formation was recently reported during skin regeneration in the African spiny mouse (*Acomys* spp.), which is the first report of blastema formation during skin regeneration in a mammal [36]. Compared to mammals axolotl full-thickness excisional skin wounds have little bleeding, and thus limited clot formation. Re-epithelialization is fast, but there is a relatively long delay in production of new extracellular matrix (ECM) [37].

2.1.3 Fish

Larval fish have the same ability of regeneration as urodele amphibians and fetal mammals, and this ability persists to some extent into adulthood. However, fish appear not to regenerate as extensively as the more advanced urodele amphibians as well as more primitive chordates [38]. Injuries to adult fish fins and heart regenerate almost perfectly, and small fish species have become important wound healing models. These include most notably zebrafish (*Danio rerio*), but also medaka (*Oryzias latipes*) [12, 39, 40]. Fin amputation is the most common injury studied in fish, but heart, nerve and eye have also received some attention [41, 42]. Fish fin regeneration proceeds through the formation of a blastema as in the amphibian limb [43], and some, but limited, dedifferentiation is reported for several tissues [33, 44]. However, the fin is devoid of muscle, and is thus not a very good model of wound healing of full-thickness excisional wounds to the trunk, such as the ones that have been the focus of this PhD study. Not much is known about skeletal muscle regeneration in fish, but very recently a few articles have

been published on the subject [12, 33, 45, 46]. Rodrigues et al [33] amputated the fin at the tail base of zebrafish larvae, thus including muscle tissue. They found that dedifferentiation was not apparent in myofibers. Gymnotiform electric fishes are emerging as interesting models of particularly nerve and muscle regeneration [46], and Unguez report similar results to those found by Richardson and co-workers. After tail amputation in the gymnotiform *Sternopygus macrourus*, epitelium covered the wound within 24h, a blastema formed within about a week and the tail regenerated its structure in three weeks, although the original size was not attained [46]. Richardson and co-workers [12] reported that dermal and epidermal regeneration is almost perfect following infliction of deep full-thickness cutaneous wounds in zebrafish. However, the affected muscle tissue did not regenerate to the same extent, and it contained more adipocytes.

The transparency of larval zebrafish makes them ideally suited to study individual cells *in vivo* 3D, and a transgenic zebrafish that stays transparent into adulthood (Pinky) has also been developed [47]. Several other transgenic zebrafish lines have been created that are useful in the study of wound healing in fish [48], such as transgenic zebrafish with traceable neutrophils [49, 50], macrophages [51] and lymphocytes [52], as well as a model facilitating spatio-temporal-specific cell ablation [53] and one for studying cell proliferation *in vivo* [54]. All in all the zebrafish has become one of the most important models for live-imaging of leukocyte migration [55], and much valuable information on wound healing will likely come from such models in the years ahead.

The larger fish species that are cultured for consumption have been the subject of some wound healing studies, but most of these were carried out in the last century at a time when molecular studies were limited in fish. Instead they often relied on histological description. Several investigations on cyprinid nerve, fin and skin regeneration date back to the 1930s (*e.g.* [56, 57]). Thus not much is known about the molecular basis of healing in these, and since size matters, also in the world of wounds, it is not ideal to extrapolate healing of the relatively small wounds in the model fish species such as zebrafish and medaka to those of larger fish.

Many consumers and recreative fishermen will have noticed the crippled fins of cultured and re-stocked salmonids. These have obviously not fully regenerated. The salmonid adipose fin has no regenerative ability and never grows back when amputated. This knowledge is actively used in fisheries management where adipose fin clipping is common practice for salmonids as a way to distinguish wild from restocked specimens when (re-)captured. However, no investigations have focused on the molecular basis for these observations.

The following sections provide first an introduction to the tissues investigated in the present PhD study, namely skin and muscle, before continuing with a description of the wound healing stages and the cells involved in these stages before finally describing some of the molecules that are important in shaping the wound healing response.

2.2 Teleost skin and muscle structure

2.2.1 Skin

Teleost and mammalian skin differs substantially. Among the most immediate differences is the production of mucus by goblet cells in the teleost skin, and the fact that the epithelium is composed of living cells, that are not cornified [5]. Since water supports an abundance of pathogens, skin and mucus provide an important barrier against infection, and it is important to quickly reconstruct this barrier following injury. Thus, re-epithelialization is fast in teleosts. Fish skin is a multipurpose organ, and in different species can serve functions such as sensory perception, respiration, osmoregulation and excretion [5]. The outer epithelial cells have microridges that have been proposed to serve various functions, including involvement in re-epithelialization after wounding [58, 59]. Adult teleost skin consists (apart from the mucus layer) of a squamous stratified epithelium with a basement membrane separating it from an underlying dermis. The dermis can be divided into an outer stratum spongiosum and an inner stratum compactum. The adult teleost epithelium is 5-10 cell layers thick. Goblet cells and melanophores [60, 61] are interspersed between the epithelial cells. Club cells (in the case of ostariophysans such as the common carp) can also be found in the epidermis in addition to other secretory cells such as sacciform cells [59]. Several types of chromatophores are also found in the skin. One of these is the melanophore. Melanophores in the skin of the cyprinid Rohu (*Labeo rohita*) are of two types: Epidermal and dermal. Epidermal melanophores have long dendritic processes, whereas the dermal melanophores have a more regular, condensed star-shape [61]. Most chromatophores, however, are located in the dermis or just below the dermis in a fatty layer (called the hypodermis in some species) containing adipocytes that are likely derived from fibroblast/adipocyte progenitors [5, 59, 62, 63]. Fibroblasts, lymphocytes, macrophages and granulocytes can all be found in variable numbers in dermis as well as epidermis in salmonids and cyprinids [5, 63-69]. Dendritic cells are common in mammalian epidermis, where they are called Langerhans cells [70]. Dendritic cells have been elusive in teleosts, and were only recently described [71, 72]. However, teleost dendritic cells may reflect a more primitive cell-type with common features of macrophages and dendritic cells, since a melanin-producing macrophage cell line from Atlantic salmon (SHK-1) express a homolog of the human dendritic cell marker CD83 [73]. The skin is also innervated and vascularized – the latter is especially apparent in mirror carp (see fig. 6) [59, 63].

2.2.2 Muscle

Piscine skeletal muscle differs substantially in architecture from their mammalian counterparts, although many of the same constituents are present, such as muscle fibers, muscle precursor cells, nerves, fibroblasts, osteocytes, adipocytes and vascular endothelial cells. Red and white muscle fibers are not interspersed as in mammals, but rather exist in discrete areas. White muscle constitutes the bulk of muscle in most fish species with an area of lateral, subcutaneous red muscle spanning the length of the fish and increasing its relative muscle contribution caudally. However, the distribution of muscle types varies somewhat with species [74]. In addition, some fish species may have an intermediate pink muscle situated between the red and white muscle. Salmonids do not have pink muscle while cyprinids do. White muscle fibers are larger in diameter than red muscle and they provide 5-10 times more power during swimming [74].

Myofibrillar genesis and composition appear conserved from invertebrates through teleosts to mammals. Myogenesis involves myoblast proliferation, migration, fusion and terminal differentiation. Muscle fibers in fish are not arranged into long bundles, but instead are short and arranged in sheets of myotomes separated by myocommata. The trunk muscles of fish are divided in left and right halves by a median septum, and in many species these are also divided in upper and lower halves. Within these blocks individual myotomes are divided by myosepta. Teleost muscle fibers are short and insert into the myosepta via tendons [74]. Adipocytes and fibroblasts are present between muscle fibers. In mammals, the ECM of muscle consists mostly of Collagen type I, in addition to several other types of collagen as well as proteoglycans, elastins, laminins and others [75]. ECM molecules have not been studied to a very large extent in fish, but it is known that while many ECM molecules (including fibrillar collagens and laminins) arose early in metazoan evolution, fibronectin (FN) arose and the collagen family expanded in the vertebrate lineage [76]. The fibrillar collagens type I and V have been described from a number of fish species, including rainbow trout (only the type I is published, but a type V homolog was found by GenBank EST database BLAST search (authors own observation)) and carp [77, 78], whereas type III, which is often present in the wound ECM of mammals and amphibians, does not seem to exist in fish [37, 79], and a GenBank teleost EST database BLAST search did not produce any clear type III homologs, but did produce homologs of several other types of collagen. However, it should be noted that Brüggemann and Lawson [80] reported the apparent presence of collagen type III in Atlantic cod muscle by using antibodies for human type III collagen.

Many fish species have a more or less indeterminate growth pattern, and continue to grow throughout life. Muscle is post-mitotic, and cannot grow by hyperplasia. Instead, normal muscle

growth in teleosts initially occurs by recruitment of satellite cells to produce new fibers, but from a certain (and apparently species-specific) size increase in muscle mass is mostly due to increase in individual fiber size, with very little contribution of new fibers [81]. For rainbow trout this shift takes place when the fish is around 50cm long. It has been proposed that the shift in muscle growth from recruitment of new fibers to growth of existing fibers is due to exhaustion of an initial pool of satellite cells [81]. These satellite cells can be considered muscle stem cells, and a body size-related exhaustion of these will result in poor muscle regenerative ability. In addition, in many fish species muscle mass fluctuates as a result of sexual maturation stage, as muscle proteins can be mobilized for energy during gonadal maturation [74]. Salmonids are a case where this muscle mobilization for energy is particularly extreme during the gonadal maturation and spawning migration. Season (light and temperature) also influences myogenesis – especially in temperate regions [74]. How these changes in muscle mass influences wound healing has not been investigated.

2.3 Stages of wound healing

Following tissue damage an intricate cascade of reactions commences aiming at containing the injury and restoring homeostasis, combatting infection, clear cellular debris and regain cellular composition and ECM architecture and, if possible, function [82]. Very short and simply put, vertebrate wound healing consist of the following sequential, but overlapping stages: 1) Hemostasis and clotting; 2) inflammation; 3) proliferation; and finally 4) remodeling. The proliferation phase includes re-epithelialization (in birds and mammals), granulation tissue formation, neovascularization, contraction, and ECM deposition (fig. 2).

In simple sterile human incisional wounds the inflammation phase typically lasts until day 4 or 5, the proliferation phase follows and lasts 2-3 weeks, and this transists into the remodeling phase, which may last for more than a year [83]. However, in reality wound healing is a highly complex sequence of events that involve a plethora of cell types, molecules and physiological processes. Excisional wounds take longer to heal and different healing phases can coexist in different locations in the wound [83].

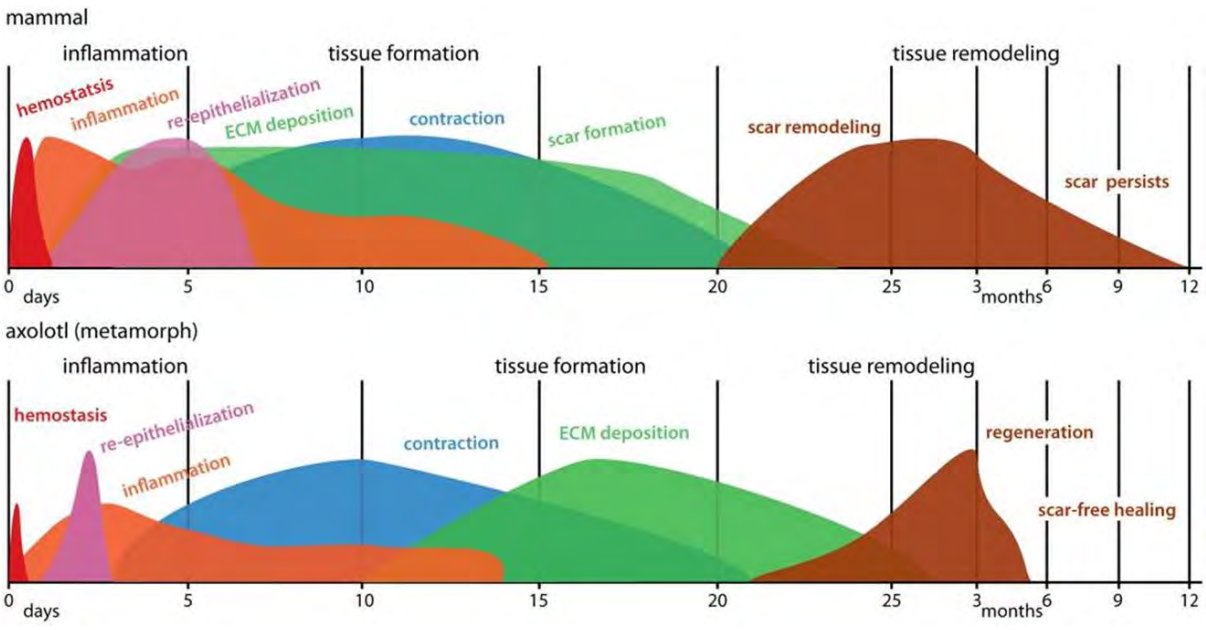


Figure 2. Graphical representation of the extent of different wound healing stages in a prototypical mammal and the axolotl. Adult mammals heal with scarring in most cases, whereas axolotl wound healing is most often scar-free (modified from [37])

2.3.1 Hemostasis and clotting

Within the first seconds and minutes after wounding – provided the wounding is severe enough to include the vascular system – blood vessels constrict to prevent excessive blood loss, platelets aggregate and blood coagulates to plug the wound and provide a matrix for infiltrating cells [84, 85]. This scenario is similar in all vertebrates.

The coagulation pathway includes several factors, but essentially prothrombin is converted to thrombin, which in turn converts fibrinogen to fibrin, which polymerizes to form a clot [84]. Most of these factors are present in zebrafish [86].

Minutes after wounding when the clot is formed, factors such as serotonin, 5-hydroxytryptamine and histamine induce the reversion from vasoconstriction to vasodilation and reversible opening of the junctions between endothelial cells to allow the diapedesis of myeloid cells into the wound site [87]. Blood vessels are stimulated by proinflammatory cytokines released from the wound to increase expression of adhesion molecules (selectins and ICAMs) essential for leukocyte diapedesis [88]. Bacterial products can accelerate the attraction of neutrophils to the wound.

In mammals the aggregated platelets degranulate, thereby releasing factors such as PDGF, which amplifies aggregation and clotting, and attracts and activates inflammatory cells, endothelial cells and fibroblasts, which also themselves produce PDGF [84, 89]. PDGF stimulates fibroblasts to secrete non-collagen ECM and collagenases such as MMP13. It also stimulates integrin expression. On the other hand, platelets also release the profibrotic cytokine TGF-β1, which stimulates collagen synthesis and decreases ECM degradation [84].

Despite the apparent similarities between mammalian and piscine blood clotting and the factors released (*e.g.* PDGF) by their respective platelets or thrombocytes, there is no significant formation of an external blood clot in zebrafish or axolotl following cutaneous injury [12, 37]. Consequently, amphibians and fish have no eschar covering the healing wound as in higher vertebrates, and an attempt to block any clotting with warfarin did not affect healing in zebrafish [12].

2.3.2 Initiation of inflammation

The initial response to wounding is likely multifaceted, but one possible pathway was recently proposed based mainly on findings from the worm *Caenorhabditis elegans* and zebrafish. These show that a calcium wave, which may be caused by changes in mechanical tension or electrical fields at the wound site, is an immediate response to damage [15, 90-93] and H_2O_2 produced by

the NADPH oxidase Duox minutes after wounding forms a gradient emanating from the wound, which provides the initial signal to attract leukocytes [93, 94]. The reactivity of H_2O_2 makes it a powerful signaling molecule through protein modification not only in the initial phases of wound healing [82]. Since Duox is regulated by calcium it was proposed that these two events were connected [82, 92], and this was recently confirmed [95]. The SRC family kinase (SFK) Lyn functions as a redox sensor for H_2O_2 in leukocytes. SFK and calcium signaling is important for epimorphic regeneration in zebrafish fin amputation studies [93].

Inflammasomes are multimolecular complexes that stimulate inflammation through the activation of caspase-1 (ICE), which in turn cleaves proIL-1β and other proinflammatory cytokines to their active form [85, 96]. However, pro-IL-1β released from necrotic cells can also be cleaved to its active form by neutrophil-derived proteases present at the wound site [97, 98]. The most studied inflammasome is NLRP3 [85, 99], but even so the exact mechanisms by which the NLRP3 inflammasome is activated in mammals is still unclear. It is known, however, that the assembly requires the presence of reactive oxygen species (ROS) such as H_2O_2 [85, 100] in addition to other triggers such as DAMPs or PAMPs [101, 102].

Pathogen-associated molecular patterns (PAMPs) signal through pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs) and receptor for advanced glycation end products (RAGE), which are likewise receptors for many damage-associated molecular patterns (DAMPs) [103]. Tissue damage caused by pathogens or by sterile means thus has different triggers, but ultimately converges in a similar inflammatory response [104].

TLRs are the most potent inducers of the inflammatory response, and the IL-1 receptor and TLRs share many signaling components [105]. Most mammalian TLRs have been discovered in fish, although some are absent in some taxa [106], and in addition some fish-specific TLRs have been discovered (TLR14 and TLR19-26) [107-109].

It appears that an NLRP3 ortholog is not present in fish [107, 110]. However, the functional response to injury is similar in all vertebrates, although it is unclear if inflammasomes are involved in piscine pyroptosis and inflammation [111].

Inflammation is an important defense against invading bacterial pathogens, and many pathogens produce substances that interfere with inflammasome activation [99].

PAMPs, DAMPs, MAMPs, RAMPs, DAMPERs and alarmins

Since PRRs recognize ligands that are not restricted to pathogenic bacteria, but also commensals, MAMP (microorganism-associated molecular pattern) has been proposed as a more suitable name for these molecules [112]. Similarly, endogenous molecules that are released concomitantly with DAMPs, but which have anti-inflammatory properties (including some heat-shock proteins (HSPs)) have been recently described and termed resolution-associated molecular patterns (RAMPs) or DAMPERs [113, 114]. The term alarmin is sometimes used for an endogenous molecule that triggers inflammation. In that terminology alarmins and PAMPs are considered subsets of DAMPs [115]. However, the more prevailing terms PAMP (for prototypical exogenous molecules derived from microorganisms) and DAMPs (for endogenous molecules released from necrotic cells) will be used in this thesis.

Most DAMPs are intracellular molecules that are released from necrotic cells. These include high mobility group B1 (HMGB1), S100 proteins, heat shock proteins, the interleukin (IL)-1-family members IL-1α and IL-33, ATP, uric acid and dsDNA, but some ECM fragments also function as DAMPs [82, 104, 116]. In addition, many of these DAMPs are not exclusively released from necrotic cells, but also actively from cells under homeostatic conditions [117-121].

HMBG1 has become the prototypical DAMP, and it is also present in fish [122]. On the other hand, IL-1α and IL-33 appear absent [123].

β-glucans are immunomodulatory PAMPs

β-glucans are a heterogeneous group of glucose-based polysaccharides that perform structural functions in a wide array of plants, algae and fungi. They are known for their immunomodulatory properties, and their effect on properties of the fish immune system has been documented in a number of studies (see [124] for a review).

In mammals, Dectin-1 is the main β-glucan receptor. However, Dectin-1 is apparently not present in fish [107]. Other receptors for β-glucans in mammals include complement receptor 3 (CR3 or CD11b/CD18, present in fish [125, 126], lactosylceramide (present in fish [127, 128]) and CD5 (not described from fish [129, 130]). CR3 may be at least partly responsible for β-glucan recognition in carp and channel catfish [131, 132]. Nothing more is known about how β-glucans are recognized in fish, but fish have a different PRR repertoire than mammals and thus likely recognize β-glucans differently [107].

β-glucans have mostly been delivered in feed, and with apparent effect [124]. Ramesh and Maridass [133] tested the effect of adding chitosan to the feed of common carp for 4 weeks prior to full-thickness wounding. Carp fed chitosan-supplemented feed had faster wound

healing (re-epithelialization, contraction, angiogenesis and formation of connective tissue). It is important to realize that immunostimulation is not constant and that timing is important. Feeding live yeast to gilthead seabream resulted in very different gene expression 2 and 4 weeks after start of yeast feeding, with a general upregulation of immune-relevant genes at week 2 and a downregulation at week 4 [60].

Others have injected β-glucans and found a positive effect by increased resistance to infection with pathogenic bacteria [134-136]. In carp, higher alternative complement pathway activity and increased phagocytic cell activity have been reported following injection [135-137]. Injection of carp with β-glucans reduced the number of circulating eosinophils and basophils in blood, and at high concentrations these cell types disappeared from blood entirely. Conversely, monocytes and neutrophils increased in number, while lymphocyte numbers were relatively constant [138].

Very recently it was reported from carp that β-glucans protect NET degradation by the fish pathogenic bacterium *Aeromonas hydrophila* [139] and that β-glucans stimulate a fast and strong respiratory burst response in carp head kidney leukocytes, compared to a low but prolonged effect of DAMP stimulation [140].

In **Paper I** we report for the first time an effect of β-glucan bathing on wound healing in common carp, and in **Paper III** we show no effect on wound healing in rainbow trout.

2.3.3 Resolution of inflammation

Regeneration usually coincides with no or limited inflammation and excessive scarring conversely coincides with an exacerbated inflammatory response [14, 141, 142]. As one of the few exceptions, substantial inflammation can still be followed by scarless healing in zebrafish larvae and adults [12, 143, 144], and conversely *pu.1* mutants (lacking myeloid cells) regenerate their caudal fins similar to wild type fish [15, 143].

Nonetheless, any inflammation needs to be terminated for healing to proceed. Resolution of inflammation is an active process, not just a slow dissipation of inflammatory molecules. Neutrophils are a source of proinflammatory factors, and apoptosis and retrograde migration of neutrophils is an important step in the resolution of inflammation [48]. However, the facilitation of regeneration by apoptosis of neutrophils is not just due to the loss of proinflammatory cytokines and tissue destructive proteases and radicals. Cells in which the apoptotic cascade has been initiated provide proliferation signals, a phenomenon called apoptosis-induced compensatory proliferation [145]. Caspases are central executioners of

apoptosis, and caspases also induce production of prostaglandin E2 (PGE2) [146]. PGE2 and Wnt signaling are master regulators of regeneration in zebrafish [147]. Apoptosis itself is not only necessary, but also sufficient for regeneration in *Hydra* [145], and apoptosis is also crucial for regeneration in *Xenopus* larvae [148].

The resolution phase marks a switch from production of proinflammatory to anti-inflammatory cytokines and a lipid-mediator switch from proinflammatory prostaglandins and leukotrienes (derived from AA) to anti-inflammatory lipoxins (derived from AA), resolvins and protectins (both derived from EPA and DHA) [149].
In case of severely infected wounds where acute inflammation is not sufficient to clear the pathogen, inflammation persists but is now dominated by macrophages and T cells [150, 151].

IL-10 is a prototypic anti-inflammatory cytokine, and many reports describe the role of IL-10 in resolution of inflammation and prevention of fibrosis. Recently Kieran et al [152] investigated the effect of IL-10 on three mammalian species. IL-10/IL-4 KO mice had elevated inflammation and excessive scar formation, conversely exogenous application of IL-10 to wild type rat and human cutaneous wounds led to better tissue architecture with less scarring. Other anti-inflammatory molecules include IL-4, IL-13, IL-22, soluble TNF receptor and IL-1ra [153].

IL-10 and IL-22 are members of the IL-10 family of cytokines. IL-10 is produced by Th1 and CD8+ T cells, natural killer cells and dendritic cells. These also produce IL-22. In addition, IL-22 is produced by Th17, Th22 and LTi cells. IL-10 exerts its effect mainly on leukocytes, whereas IL-22 affects mostly epithelial cells [154]. IL-4 is mainly produced by eosinophils with minor contributions from mast cells, and IL-13 is produced by Th2 cells and group II ILCs [155]. However, as usual in biology, spatiotemporal context is important, and priming with type I interferons (IFNs) result in proinflammatory properties of IL-10 and IL-22 [154].

2.3.4 Wound closure: Re-epithelialization and wound contraction

The wound closes by a combination of re-epithelialization and contraction. In many aquatic organisms the epidermis rapidly seals the affected area to regain osmotic homeostasis.

Embryonic wound epithelia do not migrate, and show no signs of lamellipodia or filopodia [20]. Instead, epithelial wound closure happens by a different mechanism in which actomyosin cables connect the cells of the leading edge and draw on them in a purse-string fashion to close the wound [156]. This has been demonstrated in invertebrates as well as vertebrates [19].

In adult mammals, epithelial cells start migrating over the wound as a sheet (epiboly), and they generally do not start proliferating until continuity of the epithelial layer is achieved. FN and tenascin-C (TN-C) function as guides over which the epithelial cells migrate [84].

In the axolotl, wound re-epithelialization is faster than in mammals. There is a lag phase of only 1-2h before keratinocyte migration starts, whereas this phase can be more than 24h in mammals [14]. The exact mechanisms for this fast re-epithelialization are not known, but it is known that keratinocyte growth factor (KGF) and FN are important and that expression of MMPs such as the stromelysins and MMP9 is highly elevated in the migrating epidermal front [14, 37].

The wound epithelium does more than just seal off the wound from the environment. It is also actively phagocytic [157] and provides important cues for regeneration. If autologous skin is grafted onto an amputated newt limb it fails to regenerate [15, 32].

Skin wounds are also covered rapidly by migrating epidermal cells in adult as well as larval fish. After metamorphosis the fish epidermis becomes multilayered and epithelium covers the wound by a combination of migration and proliferation of epidermal cells [158]. Migration is much faster in epidermal wounds than in full-thickness wounds including epidermis as well as dermis. In the case of the former, migration rates of up to 0.4mm per hour have been reported [159]. In plaice (*Pleuronectes platessa*) larvae, the rate of migration increased with temperature and to some extent also developmental stage [159, 160]. *In vitro* migration rates of epidermal cells from several fish species are also temperature dependent, and reach a maximum migration rate of 2mm per hour at their optimal temperature in some species [161]. In the cyprinid Rohu (*Labeo rohita*) 5mm wide and 2-3mm deep cutaneous wounds had fully re-epithelialized after 4-6 hours (at 25°C) [61].

Contraction takes place later, and is due to the action of fibroblasts and myofibroblasts applying contraction force to the extracellular matrix through mainly α-smooth muscle actin (α-SMA) [84].

2.3.5 Granulation tissue formation and neovascularization

In mammalian wound healing inflammation, re-epithelialization, granulation tissue formation and neovascularization coincide to a large extent [162]. However, in fish, re-epithelialization precedes granulation tissue formation.

Hyaluronic acid (HA) is a large glycosaminoglycan that together with fibrin form the main part of the initial provisional wound matrix in mammals [84]. HA suppresses the expression of other ECM molecules such as collagen type I and III and FN by fibroblasts [163]. However, fibroblasts later digest HA using hyaluronidase. The initial fibrin-rich wound matrix is replaced by a new matrix called granulation tissue due to its granular appearance. Granulation tissue is especially rich in FN, but TN-C and collagen is also part of this matrix. Initially collagen is mainly in the form of type III, which is a makes a thinner, more pliable fiber than type I, which is produced later [84]. Granulation tissue is considered responsible for the wound contraction observed during the proliferation phase in adult wound healing [20]. After wound contraction only little TN-C and FN remain [164]. Granulation tissue also seeds the ground for the process of re-epithelialization in mammals [20].

In zebrafish, re-epithelialization of cutaneous wounds takes place within hours, whereas granulation tissue does not start to form until day 2 after wounding, and it reaches its greatest extent after 4 days. This granulation tissue contains type I collagen, FN and TN-C. Neovascularization lags slightly behind granulation tissue formation, and only starts to be recognizable by day 4, with considerably more new vessel apparent at day 6 and 8 [12].

Oxygen is scarce in wounds due to disruption of the vascular system and the high oxygen consumption. Low oxygen levels stimulate proliferation via growth factors. At the same time lactate levels increase, which stimulates the production of enzymes involved in collagen synthesis [87]. It is important that the granulation tissue becomes properly vascularized as myoblasts are unable to proliferate or differentiate more than 150μm away from a blood vessel [165]. Vascularization of wound tissue may occur by sprouting from existing blood vessels (angiogenesis) or from the formation and fusion of new vessels (neovascularization). FGF-2 and VEGF are important growth factors promoting angiogenesis [19]. These are expressed by macrophages and damaged epithelium, and FGF-2 also by endothelial cells and nerves [19, 162]. FN and NO also stimulate blood vessel formation [82, 162]. TGF-β on the other hand promotes endothelial quiescence and thus counteracts the effects of FGF-2, VEGF, FN and NO [166]. Once the wound has contracted and sufficient amounts of new ECM have been produced the granulation tissue transist into more mature tissue by apoptosis of endothelial cells and fibroblasts and by remodeling of the ECM.

2.3.6 Remodeling

The ECM is constantly being remodeled during homeostasis, but the pace picks up in wounds [167]. There must be a delicate balance between proteases, their inhibitors and ECM molecules. Remodeling starts already from a few weeks after injury, but may last for years. During

remodeling of muscle ECM molecules that form the provisional matrix (such as collagen type III and FN) are gradually replaced by collagen type I. At the same time fibers are aligned along the lines of tension instead of the haphazard arrangement during the earlier stages of wound healing. Wound strength increases rapidly during the first weeks of remodeling, but only slowly afterwards, and it never reaches more than around 80% of the original strength in mammals. LOX is the major collagen cross-linking enzyme. Cross-linking of collagen fibers increases wound tensile strength [84].

Once the provisional matrix has been replaced by a collagen-rich matrix collagen-production returns to normal. Healed tissue is relatively acellular, and apoptosis is important event during the remodeling stage as it is for the resolution of inflammation. However, instead of neutrophils, the apoptotic cells in the remodeling phase are fibroblasts, myofibroblasts and endothelial cells. Blood vessels are abundant in granulation tissue, and endothelial apoptosis is important for vascular regression [88, 162]. Failure of fibroblasts and myofibroblasts to undergo apoptosis leads to extensive ECM deposition and thus scarring [168]. In open wounds in the rat, myofibroblast as well as endothelial cell apoptosis peaked around day 20 – five days after the wound had closed. Interestingly, artificial and premature closing of the wound by skin grafting induced myofibroblast apoptosis in a matter of hours [83].

2.4 Cell types involved in wound healing

In normal mammalian wound healing neutrophils start invading wound from 2h post-wounding and their numbers peak 6-24h post-wounding. Macrophages and fibroblasts invade the wound after 24h with a peak after 4 days, but some persist well into the remodeling phase [83, 165]. Eosinophils infiltrate the wound at an intermediate time between neutrophils and macrophages [169]. After about two days the macrophage population shifts from M1 to M2. After about a week fibroblasts start to differentiate into myofibroblasts [170]. Muscle progenitors also start invading the wound around 24h post-wounding [165].

The sequence and extent of cell infiltration is species specific in fish, and the timing also depends on temperature. The studies by Iger and Abraham [157] and Mawdesley-Thomas and Bucke [171] provide examples of cell infiltration into different wound types in cyprinids kept at around 20°C. The sequence of cell infiltration into the wound largely resembles that of mammals with the exception that basophils are the most abundant leukocyte from a few hours and up until 1-2 days post-wounding [157].

The following section describes how the cell types found in fish wounds may contribute to the healing process. Most of the information comes from mammals.

2.4.1 Thrombocytes

Thrombocytes are the lower vertebrate equivalent of platelets. Platelets are initially involved in hemostasis and thrombosis, by aggregation and secretion of coagulation factors [172]. Human platelet and zebrafish thrombocyte hemostasis is quite similar [86, 173].

Platelets contain a plethora of factors (including PDGF, TGF-β, FGF-2, IL-1β and FN) that are stored in granules and released following activation [172, 174]. However, these factors are also produced by other cells, and platelets are not essential for normal adult mammalian wound healing [20]. The integrin α-chain CD41 is a marker for platelets, and it dimerizes with an integrin β-chain (CD61) to form the functional integrin CD41/CD61, which is the most important receptor involved in platelet aggregation, and the most abundant molecule in the cell membrane of human platelets [175]. CD41 expression during wound healing in rainbow trout is described in **Paper III**.

Thrombocyte function has not been much studied in fish. Channel catfish (*Ictalurus punctatus*) thrombocytes were found to likely aggregate due to the binding of CD41/CD61 homologs [176]. In humans and mice as well as zebrafish, two $CD41^{+}$-populations exist ($CD41^{high}$ and $CD41^{low}$).

Lin et al [177] cloned zebrafish CD41 and created a CD41-GFP-transgenic zebrafish. In humans and mice these two populations have been shown to consist respectively of platelets and very early hematopoietic progenitors in which the *cd41* gene is silenced as cells develop. This is similar in zebrafish, with the $CD41^{low}$ cells having hematopoietic properties when grafted into irradiated zebrafish, while the $CD41^{high}$ cells do not [178]. Rainbow trout thrombocytes also express cytokines such as IL-1β, TNF-α and TGF-β and produce eicosanoids just as in mammals [179].

However, there are also numerous differences between mammalian platelets and piscine thrombocytes. Piscine thrombocytes are nucleated cells that are morphologically very similar to lymphocytes. Platelets are much more numerous in human blood than thrombocytes are in fish blood. A platelet count of more than 10^5 μL^{-1} is normal in human blood, while teleost thrombocytes usually number less than 10^4 μL^{-1} [180, 181].

In addition, teleosts lack an ortholog of the collagen receptor GVPI, which is important for activation and aggregation of mammalian platelets. However, a different receptor performing similar functions was recently described [182, 183]. This receptor was also expressed on erythrocytes in some species, and these may thus contribute to hemostasis in fish.

Thrombocytes also share some of the same features of B1 lymphocytes, such as being phagocytic [184-188]. Rainbow trout thrombocytes additionally express MHC class II, TAP1 and TAP2 (involved in antigen presentation), and CCR7 (which in mammals is involved in the homing of T cells to lymph nodes, and probably have a similar function in rainbow trout despite the lack of true lymph nodes [189]), and thus potentially participate in adaptive immune responses [190, 191]. Piscine nucleated thrombocytes could be speculated to persist for some time in the wound, and to continue to secrete cytokines and growth factors, as well as actively helping clear the wound of pathogens and cell debris by phagocytosis.

2.4.2 Granulocytes

In mammals the granulocytes are divided into neutrophils, eosinophils, basophils and mast cells.

In fish, the granulocyte nomenclature is not so fixed, and the staining characteristics and composition of granulocytes vary considerably between species [69, 192, 193]. For example, in carp wounds basophils are one of the most prominent infiltrating cell types [157], whereas basophils are rarely observed in salmonids altogether [194]. In salmonids cells with characteristics of both mast cells and eosinophils (coined MC/EGCs by some authors) are

common in mucosa-associated lymphoid tissue, and are recruited to inflammatory sites [69, 195].

Apart from neutrophils the involvement of granulocytes in wound healing has been under-studied in mammals as well as fish, and these are more commonly associated with allergy and anti-parasitic responses [196, 197]. However, anti-parasitic responses are also aimed at containing the concomitant tissue damage caused by large parasites, and may thus have important functions in wound healing. In fact, it has been proposed that Th2 immunity evolved mainly as a repair response to the tissue destruction caused by metazoan parasites [198].

Neutrophils

The neutrophil is one of the main inflammatory cells. Neutrophils circulate in blood, and are trapped together with platelets and erythrocytes in the blood clot [142]. Neutrophils are also the first leukocytes to actively infiltrate the mammalian wound in large numbers [142]. Here they secrete eicosanoids, reactive oxygen and nitrogen species, proteases that debride the wound and a range of chemokines and cytokines that attract more neutrophils as well as macrophages and T cells to the wound site [142, 150, 199, 200]. It was recently shown in mice that the DAMP IL-1α released from necrotic cells specifically recruits neutrophils, whereas IL-1β recruits macrophages [201]. Neutrophil secretion factors also promote angiogenesis and the migration and proliferation of keratinocytes and fibroblasts [202].

Another important part of neutrophil function is programmed cell death. Initially when entering the wound, neutrophils downregulate proapoptotic and upregulate anti-apoptotic genes [202], but at later stages apoptosis is an important part of neutrophil function. One apoptotic pathway used by neutrophils is NETosis [203, 204]. NETosis results in the release of a so-called neutrophil extracellular trap (NET) consisting of decondensed chromatin. This NET is decorated with proteases, histones and myeloperoxidase, all of which have a bactericidal effect [205]. NETs may also have a function in homeostasis as Nox-2 deficient mice (*i.e.* NETs cannot be formed) are more prone to lupus than wild-type mice [206]. However, neutrophils do not all undergo apoptosis and/or are cleared by macrophages and fibroblasts at the wound site. Neutrophils also actively leave the wound site, and reversion of chemotaxis to chemorepulsion in neutrophils in response to an unknown signal is important in resolution of inflammation [207]. In fact, neutrophil emigration appears to be more common than apoptosis during the resolution phase in zebrafish [55]. Neutrophils are even reported to migrate from virally infected dermal sites to the bone marrow to help prime CD8+ T cells and a range of other functions of neutrophils are emerging [208, 209].

Morphologically fish neutrophils of some species differ from those of mammals, since not all fish species have neutrophils that are polymorphonuclear. Nevertheless these seem to function in a very similar manner [210]. They typically start arriving at the site of injury after about an hour and their numbers peak after 48h [211]. Fish neutrophils are phagocytic, but unlike their mammalian counterpart they have relatively poor bactericidal activity compared to macrophages [181, 211]. Surprisingly non-specific cytotoxicity has been reported from rainbow trout neutrophils [212]. NET formation was also described from fathead minnow (*Pimephelas bromelas*), zebrafish and carp, and it is probably found in all fish as the principle is an ancient defense strategy also found in invertebrate hemocytes [213-215].

Non-neutrophilic granulocytes

Mammalian mast cells have abundant granules of histamine, serotonin and heparin. These have several biological effects including vasodilation and chemoattraction of neutrophils [216], and can be released upon stimulation with or without the concomitant release of lipid mediators, cytokines and growth factors. However, mast cells can also respond by chemokine synthesis in the absence of degranulation [217]. Mast cells bind and respond to the DAMP IL-33 through ST-2, however, mast cells do not respond to other DAMPs such as HMBG1, adenosine or uric acid, and in fact IL-33 appeared to be the sole factor from necrotic cells that stimulated a response from mast cells [218]. This response was proinflammatory through the release of TNF-α, IL-6 and leukotrienes. IL-33 does not trigger degranulation on its own, but potentiates the response to degranulation signals. On the other hand, mammalian basophils release the Th2 cytokines IL-4 and IL-13 when stimulated with IL-33 [217].

Recently it was discovered that eosinophils (and to a lesser degree mast cells) are the main IL-4 producing cells in regenerating muscles of mice [155]. In addition it was shown that *il4*$^{-/-}$*il13*$^{-/-}$ mice failed to resolve inflammation, and that inflammatory cells and debris persisted at the wound site. Thus, mast cells and especially eosinophils are important for the switch from an inflammatory Th1 to a resolving Th2 environment – at least in mice.

Eosinophils produce a range of (mainly Th2-type) cytokines, chemokines, lipid mediators and cytotoxic cationic proteins. These are mainly considered to partake in the immune response towards parasites. In addition, eosinophils produce a number of proresolving EPA- and DHA-derived lipid mediators, which have only been discovered within the last decade [169]. These include resolvins, protectins, and maresins. They inhibit neutrophil infiltration and stimulate macrophage phagocytosis of apoptotic neutrophils. Depletion of eosinophils or knock-out of 12/15-lipoxygenase prolongs the resolution phase [169].

Piscine mast cells contain granules with enzymes such as phosphatases and lysozyme as in mammals, but no serotonin. Histamine and piscidins (antimicrobial peptides) are only found in mast cells of the advanced order perciformes [219, 220], and thus neither in cypriniformes, nor salmoniformes. Teleosts also lack an IgE and IL-33 homolog [123], although the IL-33 receptor ST2 is apparently present [221]. Ligand binding to IgE and ST2 is known to activate mast cells in mammals. Nonetheless, Mulero et al [220] found perciform mast cells to functionally respond to stimulation much like mammalian mast cells. Likewise, Balla et al [222] found zebrafish eosinophils to share a number of features with mammalian eosinophils, including similar responses to stimulation. However, neither IL-5 nor its receptor is apparently present in the zebrafish genome, and this signaling pathway is central for mammalian eosinophil function [197]. Another difference is that eosinophils are not very common in zebrafish compared to mammals and salmonids, and appears to play a minor role [48, 222], whereas basophils are abundant in wounds of common carp [157, 223].

In addition, a unique and enigmatic cell type called the rodlet cell is only found in fish. It somewhat resembles MC/EGCs, but has characteristic club-like inclusions. Rodlet cells are often found alongside MC/EGCs during parasitic infections [69, 192, 224, 225]. With the theory of the evolution of eosinophilic granulocytes to maintain tissue homeostasis during the tissue destructing infection with large metazoan parasites in mind, they could thus be speculated to perform an as yet uninvestigated role in piscine wound healing.

2.4.3 Macrophages

Macrophages are key orchestrators of wound healing through phagocytosis of apoptotic cells as well as secretion of growth factors and cytokines. They are arguably the most studied cell type in relation to wound healing.

Macrophages at the wound site can derive from either local tissue macrophages residing in the vicinity of the wound, or from circulating monocytes. The former perform homeostatic functions and are thus anti-inflammatory. It was recently discovered that tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes, and that monocytes and tissue-resident macrophages should be considered independent lineages [226]. However, at least in mammals, tissue-resident macrophages are relatively scarce, and apparently only make a minor contribution to wound healing [151].

Following damage monocytes extravasate from the circulation into the wound site and transform initially into M1 macrophages, which facilitate the eradication of any microbial

organisms that might enter the wound. However, this phenotype is tissue destructive, and it is important for healing that the wound milieu changes to Th2/M2 [227]. Under normal wound healing conditions in mammals, this shift to the resolving M2c-type CD163+ macrophages takes place already within two days [199], and after less than a week macrophage numbers start declining in the wound [165].

Macrophage polarization is reversible, but it is not known exactly to what extent the transition of the wound macrophage population is due to a change in phenotype of the macrophages already in the wound, or due to M2 polarization of newly arriving monocytes, although the former scenario may be the predominating [151, 228]. Mainly M2 macrophages are responsible for efferocytosis of apoptotic and necrotic cells [229], and it appears that M1 macrophages are themselves actively triggering their change to M2 when they phagocytose apoptotic cells [151]. Macrophage polarization is not only dependent on the chemical environment, but also on physical structure of tissue as shown by cell culture on electrospun scaffolds. A loose tissue with large pores favors M2 polarization, whereas smaller pores favor the M1 phenotype [227].

The M1 phenotype is proinflammatory and is induced by IFN-γ and PAMPs [230-232]. M1 macrophages typically produce proinflammatory cytokines such as IL-1β, IL-6 and TNF-α as well as reactive oxygen species, nitrogen intermediates (produced by iNOS) and a number of proteases [233, 234].

The M2 phenotype is mostly anti-inflammatory and quite diverse. M2 macrophages are usually subdivided into M2a, M2b and M2c macrophages, which are induced by IL-4/IL-13, immune complexes/TLR agonists and IL-17/IL-10/glucocorticoids respectively [227, 234, 235]. The M2c phenotype is the one mainly associated with CD163 expression as well as clearance of apoptotic neutrophils, and thus with resolution of inflammation [235, 236]. Murine M2 macrophages are characterized by expression of Ym1, arginase 1, scavenger (*e.g.* CD163) and mannose receptors and anti-inflammatory cytokines (*e.g.* IL-10, IL-1ra, TGF-β), and also MMP9 and insulin-like growth factor (IGF-1) [230, 232, 237]. Arginase 1 competes with iNOS for L-arginine and thus suppresses inflammation [238]. However, the markers used to characterize these macrophage phenotypes vary with species [239], and human M2 macrophages do not upregulate Ym1 or arginase-1 [239, 240]. Regardless of polarization, macrophages are able to attract myogenic cells by release of HMGB1 and TNF-α (in the case of M1) or MMP-9 (in the case of M2) [241].

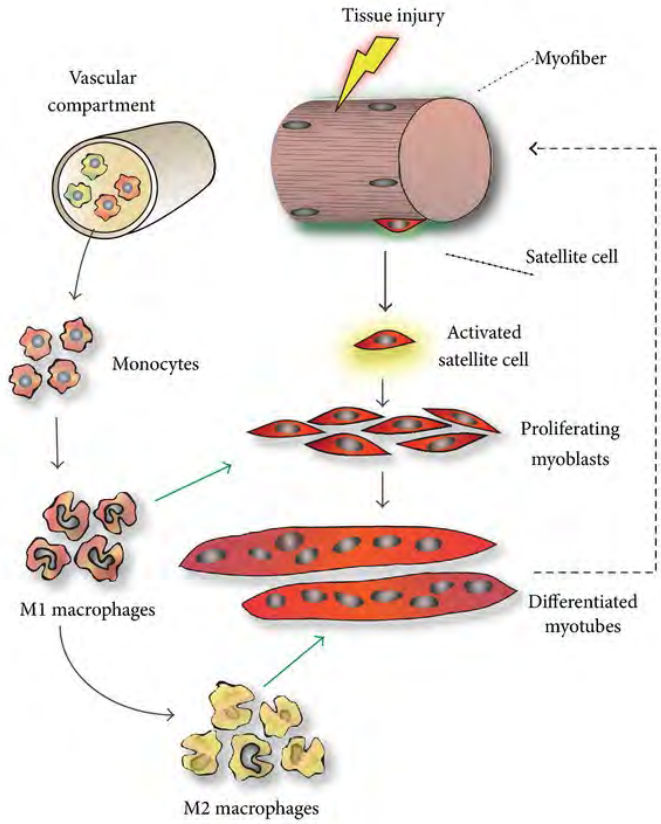


Figure 3. Illustration of the involvement of macrophages in healing of wounds to the muscle. M1 polarized macrophages found in the inflammation phase release factors that stimulate myoblast proliferation. M2 polarized macrophages are found at later wound healing stages and the promote myoblast differentiation and fusion (from Kharraz et al 2013).

In the absence of macrophages in the early stages after injury, adult mammalian muscle show defective healing, and it is thought that this is due to cross talk between macrophages and pericytes/satellite cells (fig. 3) [142, 153, 232, 242], and it was recently shown that macrophages were absolutely required for limb regeneration during the first 24h after limb amputation in axolotl [243]. However, the gene expression dynamics of wound macrophages in the axolotl differs somewhat from the mammalian system, and pro- and anti-inflammatory cytokines are induced simultaneously during this time. Macrophage depletion decreases MMP9, TGF-β and FN expression in the wound. However, re-epithelialization in the axolotl is not affected by macrophage depletion [243] as it is in mammals [242]. Later depletion of macrophages (after blastema formation) resulted only in delayed limb regeneration.

Fish macrophages seem to function much like in mammals, but the key mammalian myeloid growth factors IL-3 and GM-CSF seem not to be present in fish [244]. CSF-1R has recently been shown to be a pan-macrophage-lineage marker in goldfish [245]. Macrophage polarization in fish has not been extensively studied, but seem to functionally resemble the mammalian paradigm [246], although the PRR repertoire in fish is quite different from mammals, and also

between different fish taxa [106-108, 247], and the molecular pathway and level of response to PAMPs such as LPS (which is commonly used to induce the M1 phenotype) differs [246].

2.4.4 Lymphocytes

The lymphocyte lineage has classically been associated with adaptive immunity with the exception of natural killer cells. Several subsets of B cells (*e.g.* B1a, B1b and B2) and T cells (*e.g.* Tc, Treg, Th1, Th2, Th17 and Th22) have been described. T and B lymphocytes are scarce in uninfected mammalian wounds, which may explain why the involvement of these cell types in wound healing is understudied [248]. However, normal human skin harbors intraepithelial lymphocytes (IELs) of which most are $CD8^+$ and γδ T cells [181, 249]. Innate-like γδ T cells are involved in wound healing and skin homeostasis [250], and T cells persist to become the most numerous leukocyte during the tissue maturation phase of human skin wound healing [142]. γδ T cells constitutively express CCR6 and RORγt, and IL-1R activation leads to transcription of the cytokine IL-17A [249].

Recently it was also shown that B lymphocytes participate in wound healing, although not locally. Instead circulating autoantibodies bind to targets at the wound site and enhance cutaneous wound healing in mice, presumably by enhancing phagocytosis. Interestingly, these antibodies were not IgM, which is the most common isotype of innate antibodies especially in lower vertebrates [251, 252], but IgG1 [248].

Within the last decade the separation between adaptive and innate branches of the immune system has faded. Several innate lymphoid subsets (innate lymphoid cells; ILCs) without variable receptors or innate-like lymphoid cells (*e.g.* innate natural killer T (iNKT) cells and mucosal-associated invariant T cells (MAITs)) with limited variability of their receptors have been discovered in mammals, and many of these appear to be involved in wound healing [249, 253, 254].

Considering the layering hypothesis [255, 256] and the primitive adaptive immune system fish (teleosts respond relatively poorly to immunization, resulting in low affinity antibodies with limited affinity maturation and a poor secondary response [257-261]), it is plausible that lymphoid cells with more innate properties like the recently discovered mammalian ILCs will also be discovered in fish.

Due to the recent discovery of ILCs, the terminology has not yet been fixed, but it was recently proposed to group them into three groups; I (including NK cells), II and III. Group I ILCs produce mainly Th1-type cytokines, group II ILCs Th2 cytokines and group III Th17 cytokines [262].

Group II and III ILCs are involved in parasitic defenses and tissue healing at mucosal surfaces through expression of IL-4, IL-13 [263] and IL-22. In addition, group III ILCs also produce IL-17A [249, 262], which promotes M2c macrophage polarization [264] and has antifibrotic properties by downregulating connective tissue growth factor (CTGF) and collagen type I [265]. IL-17A is also produced by γδ T cells, Th17 cells and NKT cells [265].

Group II ILCs thus could play a role in the switch from a Th1- to a Th2-dominated milieu during the resolution phase of wound healing. Group II ILCs (nuocytes and natural helper (NH) cells) express ST-2, which is a receptor for the DAMP IL-33. Nuocytes were originally described as IL-25 (IL-17E) responsive and IL-4, -5 and (especially) -13-producing in response to helminth infection [263]. However, nuocytes are also responsive to IL-33 and produce IL-6, IL-10 and GM-CSF [266]. NH cells are mostly found in adipose tissue and produce IL-5 and IL-13 in response to stimulation with IL-33 (in addition to IL-2, IL-7 or thymic stromal lymphopoietin (TSLP)). Using intravital microscopy it was established that a group II ILC subset in mouse dermis are found in contact with mast cells. This subset potentially modulates mast cell activation through paracrine IL-13 secretion [267]. They thus seem implicated in switching the area of tissue damage from a Th1 to a Th2 response [249].

For several decades lymphocytes have been known to be present in fish skin and to infiltrate wounds [64, 157, 171]. However, lymphocytes have not been specifically studied in relation to wound healing in fish, and studies of lymphocyte subsets in fish have altogether been hampered by a lack of monoclonal antibodies (MAbs) and other molecular tools. It is likely in my opinion that these lymphocytes may include as yet undescribed piscine ILCs.

B cells, CD4+ helper and CD8+ cytotoxic T cells and some innate-type lymphocytes are described from fish. B cells can be subdivided on the basis of their B cell receptors, but no distinction between B1a, B1b and B2 subsets as in mammals have been reported, although the properties of piscine B cells resemble those of mammalian B1 cells [268]. Likewise, no T helper cell subsets are firmly established in fish, although based on cytokine and transcription factor expression patterns there seems to be T_{reg}, Th1, Th2 and Th17-type responses [269, 270], as well as γδ T cells [271, 272]. The innate-like lymphocytes in fish are described as natural killer-like cells or natural cytotoxic cells (NCCs) [269]. NCCs are described from rainbow trout [273], but innate-like lymphocytes have been mostly investigated in the channel catfish, where several subsets of NCCs are described [274].

However, in the recent few years slow advances have been made in the study of lymphocyte subsets [275, 276]. Takizawa et al [272] identified at least some of these to be $CD8^+$ and $TCR\gamma^+$ lymphocytes in rainbow trout gills using an anti-CD8 MAb. However, these were not very

common in skin [269]. In addition, Dixon et al [277] characterized CCR6 from rainbow trout and found constitutive expression in gills, thymus and PBLs. A RORγt homolog is induced by LPS stimulation in the skin of rainbow trout, suggesting a role in bacterial protection [278], and the transcription factor GATA-3 is expressed in Atlantic salmon skin [279]. These transcription factors characterize ILC class III and ILC class II, respectively in mammals [262, 280], although GATA-3 is not so restricted in its cellular expression.

2.4.5 Fibroblasts and myofibroblasts

Following injury, and especially injury with volumetric loss, the production of new extracellular matrix molecules is essential for wound healing. Fibroblasts and myofibroblasts secrete most of the ECM molecules (including collagens, FN and TN-C) and proteases (including MMP9 and MMP13) required for new matrix production and remodeling [168, 281, 282]. They also secrete a wide range of cytokines and growth factors, such as FGF-2, TGF-β and VEGF that promote re-epithelialization, vascularization and granulation tissue formation [283]. IGF-1 secreted by M2 macrophages blocks the apoptotic pathway in fibroblasts and thus leads to fibroplasia, and in non-infected wounds fibroblasts are the main cell type by day 3-5 after wounding [83].

The fibrocyte is a circulating fibroblast precursor [281]. Fibroblasts are spindle-shaped cells widely distributed in especially connective tissue [281]. They express the cytoskeletal component vimentin, but not desmin or α-SMA [281].

Myofibroblasts are phenotypically intermediate between fibroblasts and smooth muscle cells [281]. They are distinct from fibroblasts by extensive cell-matrix and cell-cell interactions as well as contractile cytoplasmic microfilaments such as α-SMA [281], α-SMA is the most commonly used histological marker of myofibroblasts, and it is expressed prior to and during wound contraction [168]. Local fibroblasts are considered the main source of wound myofibroblasts [170]. However, myofibroblasts may derive from several sources other than fibroblasts such as pericytes, epithelial cells and circulating progenitors [166, 168, 282]. α-SMA is also expressed by pericytes and is an early differentiation marker of vascular smooth muscle cells [88]. In a recent study on rat excisional muscle wounds myofibroblasts apparently solely arose from blood vessel pericytes and perifollicular dermal sheath cells [284].

Fibroblasts differentiate into myofibroblasts, only in the presence of the ED-A splice variant of FN (which is expressed by macrophages and myofibroblasts themselves), mechanical stress and TGF-β1 [170, 285].

The myofibroblast is the main cell type involved in wound contraction. Contractile forces proximate the wound edges and myofibroblasts function as clamps to hold the tissue together until remodeling has resulted in adequate strength [281]. α-SMA is not absolutely required for production of cell traction force, but α-SMA is able to generate more contraction force than other actin isoforms, and thus enhances contraction [281, 286]. In rat wounds proto-myofibroblasts appear 6 days after wounding with fully mature myofibroblasts peaking after 9 days, coinciding with the contraction phase [170, 284]. After the ECM has been reconstructed it takes over the mechanical load from the myofibroblasts and these die from apoptosis [170, 286].

To some extent fibroblasts can stand in for inflammatory cells in *pu.1* knock-out mice, which heal wounds with minimal scarring [20]. Fish fibroblasts have not been very much studies, but several fibroblast cell lines exist for fish, and appropriate stimulation of these with PAMPs (LPS) and DAMPs (cell debris and collagen motifs) show that they also produce immune-related factors [287, 288].

2.4.6 Pericytes and satellites

Pericytes (also called mesangioblasts) and satellite cells are distinct precursors important for wound healing [166, 232, 289]. Pericytes are associated with blood vessels, and are typically linked with vascular formation and function [166]. Satellite cells are situated between the muscle cells and the muscle basement membrane and are stem-like cells associated with postnatal mammalian muscle regeneration [290].

However, both these cell types constitute a very heterogeneous population, and no specific pericyte markers exist. Mesenchymal and adipose-derived stem cells also possess pericyte-like properties and these give rise to myofibers as well as other tissues, and pericytes can also give rise to myofibroblast-like cells [166]. Additionally, pericytes in the brain have been shown to attain macrophage-like properties following injury, but this has not been shown for pericytes in muscle [291]. Muscle- and bone marrow-derived mesenchymal stem cell populations contribute to the satellite cell pool [165]. Exactly how stem cells and progenitors contribute to the pool of different tissue-regenerating cells is still largely unexplored [292].

Dulmovits and Herman [166] recently reviewed the involvement of pericytes in wound healing, and this matter shall not be further elaborated here. However, after this review was published an interesting discovery was reported: It had been known for some time that myeloid cells made frequent contact with pericytes during recruitment to inflammatory loci, and it was

shown that the pericytes stimulate these myeloid cells to express prosurvival and promigratory molecules, such as MMP9 and integrins [293, 294].

Satellite cells contain very little cytoplasm and are quiescent in the absence of muscle damage. Pax-7 is a marker of muscle satellite cells. When activated (*e.g.* by muscle injury, which releases growth factors such as FGF-2 from the ECM) satellite cells start expressing muscle regulatory transcription factors (MRFs) such as MyoD, Myf-5 and myogenin [295]. This is also the case in fish [46, 165, 296-301]. Pax-7, MyoD and myogenin is expressed in the mentioned order during the transition from quiescent satellite cells to differentiated myoblasts, and the relative expression levels have been proposed to be useful in establishing the stage of muscle regeneration [300]. The myoblasts then form myotubes, which fuse with existing myofibers [165]. Olguín and Pisconti [300] provide a good and brief overview of important transcriptional pathways that regulate myogenesis through activation of satellite cells.

Inflammatory molecules such as TNF-α and IL-6 promote satellite cell chemotaxis and the former also proliferation. On the other hand, TGF-β1 suppresses proliferation of satellite cells [302]. Like other wound-infiltrating cells such as macrophages and fibroblasts, satellite cells also release gelatinases and other MMPs when entering the wound. These ECM degrading enzymes liberate growth factors to stimulate cell migration, proliferation and differentiation [165]. Myoblasts express MMP9 prior to formation of myotubes [303].

The shift from a Th1 to a Th2 environment coincides with myogenic differentiation, and FN, IL-4 and IL-10 all induce migration of myoblasts, and fusion and maturation of myotubes [165, 199, 303]. The IL-4 receptor is found on myeloid cells and common fibrocyte/adipocyte progenitors (FAPs), and it was recently shown that myeloid-specific loss of this receptor did not substantially affect regeneration, but complete loss did [155]. In the same study it was also found that FAPs were more important for clearing necrotic cell debris from the wound than myeloid cells were.

IGF-1 and hepatocyte growth factor (HGF) are main growth factors involved in regulating skeletal muscle repair, and IGF-1 is critical myoblast proliferation and differentiation and thus for muscle growth (see **section 3.2**) [165]. On the other hand, TGF-β is the factor thought to have the greatest inhibitory effect on muscle progenitor cell proliferation, recruitment and differentiation [165, 295].

Satellite cells have been discovered, isolated and studied from several fish species [297, 304]. Satellite cells and myoblasts have also been investigated in salmonids, and these largely respond to the same stimuli and in the same way as in mammals, although in some respects

they are more like fetal myoblasts [297]. In addition, several paralogs of MyoD exist in Atlantic salmon, and these are differentially expressed during different maturation stages [298].

Most fish have a relatively indeterminate growth pattern, *i.e.* they continue to grow throughout life. This is not so for zebrafish, and this may make it a good model for mammalian muscle regeneration, but peculiarly so maybe not for fish. However, the closely related giant danio (*Devario aequipinnatus*) does have an indeterminate growth pattern. By comparing these two species Froehlich et al [305] identified Pax3 and/or Myf-5 as possible factors involved in this difference in growth pattern.

2.4.7 Secretory cells

Goblet cells are the main mucus producing cells in fish. They contain a diverse range of mucins as well as immune factors [306, 307]. They are abundant in the epidermis, and apparently differentiate from cells in the lower epidermis and mature as they move towards the upper epidermis. However, we found mucin expression in healing muscle of carp two weeks after wound infliction. This could indicate that goblet cells may originate from deeper locations than epidermis (**Paper I**). When they reach the surface, the cell membrane ruptures, spilling the mucus onto the surface of the skin and the goblet cell then dies. Irritants or wounding induces secretion of goblet cell content. The secretory cells of fish are diverse, and are separated by their shape, size and staining characteristics [59, 61, 307].

The fishes in the superorder Ostariophysi have a special cell type called the club cell located in the epithelium. These were originally described as a reservoir of "fear factors" that are released by skin injury and which can be sensed by conspecifics and produce a flight response [308, 309]. However, this may just be a secondary function, and club cell constituents may be primarily involved in protection from UVB light and bacterial and parasitic pathogens [310]. Club cell function has not been investigated in relation to wounding, but Skoric et al [6] observed that common carp surviving cormorant attacks (with resulting damage to the skin and sometimes muscle) had increased number of club cells and melanocytes compared to non-injured fish.

2.4.8 Melanophores

The melanophore is one of several chromatophore types found in fish [59]. As early as the 1930s Smith (1931) noted the association of melanophores with healing skin wounds in goldfish. Since then several investigators have observed and described this phenomenon in

different fish species [6, 45, 61, 311-313]. In fact, wound hyperpigmentation is a recurring feature in the animal kingdom [314, 315]. Wound hyperpigmentation in zebrafish was recently linked to inflammation [45]. During wound healing in Rohu the chromatophores degenerate and reform [61]. However, exactly how melanophores contribute to healing is not yet clear.

For more on melanophores in wound healing see **section 3.1**.

2.4.9 Nerves

Nerves were shown to be important for salamander limb regeneration as early as 1952 [316], and in 1988 Margaret Egar showed that ectopic accessory limbs could be induced in Axolotl by surgically deviating large limb nerves from their normal path [317]. She proposed that three factors were necessary for this to occur: Wound epidermis; a source of neurotrophic factor; and a source of proliferating cells. The nerve provided the latter two and the surgical procedure the first. Since then it is becoming clear that nerves are important for epidermal dedifferentiation during wound healing the axolotl [318], and nerves supply important factors (such as FGFs and newt anterior-gradient (nAG)) during salamander regeneration [14, 32]. In vertebrates, nervous signals are not needed for the initial stages in muscle regeneration, but subsequent growth and maturation of regenerating muscle fibers require innervation [319], and loss of innervation leads to muscle atrophy [165]. Further indicating the importance of nerve signals in wound healing is the fact that naturally poorly innervated parts of the body also exhibit poor wound healing [19].

2.5 Investigated genes

Here I present the genes that are investigated in **Paper I-III**. IGF-1 and FGF-2 expression was investigated in an unpublished experiment and are instead briefly described herein (**section 3.2**). CD41 is a thrombocyte marker and was described in **section 2.4.1** on thrombocytes.

2.5.1 Interleukin (IL)-1β

Inflammation is in most situations a fundamental response to various sterile and non-sterile stimuli, and IL-1β is well-established as a proinflammatory cytokine in all vertebrates. Its expression is highly inducible after pathogenic, chemical or mechanical stimulation [123]. IL-1β and other proinflammatory cytokines increases expression of cell adhesion molecules on endothelial cells, thereby facilitating diapedesis through interaction with β1(CD18)-integrins on myeloid cells [153]. IL-1β also stimulates fibroblast proliferation [320].

Several teleost species have more than one IL-1β isoform [123]. Rainbow trout and Atlantic salmon have three, and Atlantic salmon has an additional IL-1β pseudo gene [321, 322]. Common carp has at least two [323], whereas only one has been described from zebrafish [321]. Carp IL-1β1 is constitutively expressed mainly in head kidney, whereas IL-1β2 is more broadly expressed. Stimulating head kidney cells with LPS led to upregulation of both [323]. The salmonid IL-β3 isoform also seems to play a role in inflammation [321]. Recently Chris Secombes' group compared their genetic structure and found that they clustered into two groups termed type I and II [321]. However, functionally this division is not so simple, as the expression of type II salmonid isoform 1 is more mammalian IL-1β-like than the two other functional isoforms, whereas the carp IL-1β isoforms as well as the single zebrafish IL-1β are of type I [321].

Another feature of fish IL-1βs is the lack of an apparent prototypical caspase-1 (also called interleukin-1-converting enzyme (ICE)) cut site. However, earlier indications of IL-1β activation through cleavage by caspase-1 [123, 324, 325], has recently been confirmed in a zebrafish, where it was shown that following bacterial infection IL-1β in primary leukocytes is cleaved by a caspase-1 ortholog and secreted in a manner similar to that seen in mammals [326]. On the other hand, in gilthead seabream, IL-1β processing and release is apparently caspase-independent altogether, and the authors propose that in fish IL-1β processing is coupled to its synthesis, instead of the delayed activation of proIL-1β in mammals [111].

IL-1β expression is described in **papers I-III**.

2.5.2 Inducible nitric oxide synthase (iNOS)

Nitric oxide (NO) is a small free radical produced by nitric oxides synthases. Three isoforms of NOS exist in mammals; neuronal (nNOS/NOS1), inducible (iNOS/NOS2) and endothelial (eNOS/NOS3). nNOS and eNOS are constitutively expressed, whereas iNOS is only present at low levels under homeostatic conditions [327]. iNOS is expressed by macrophages, lymphocytes, neutrophils, keratinocytes and fibroblasts after wounding [328].

NO is important for the eradication of pathogens in combination with H_2O_2, and also an important cell-signaling molecule [327]. It can also activate nociceptors and thus cause a sensation of pain [329].

Anderson [330] reported more than a decade ago that NO is important for muscle regeneration through its effect on muscle satellite cell proliferation, differentiation and fusion to form new myofibers [330]. A recent report builds on these and later results by looking at the specific involvement of iNOS in murine skeletal muscle regeneration [331]. Rigamonti and co-workers find that iNOS expression in injured muscle is mainly restricted to infiltrating macrophages, and that myogenic precursor cells of $iNOS^{-/-}$ mice fail to proliferate and differentiate [331]. These mice also have an increased infiltration of neutrophils and a persistent infiltration of macrophages. In mice, iNOS expression peaks one day after wounding, and returns to control values by day 10 after wounding [331].

The effect of wounding and PAMP bathing on iNOS expression in rainbow trout and on respiratory burst activity in carp head kidney macrophages is reported in **Paper III** and **Paper I**, respectively.

2.5.3 Transforming growth factor (TGF)-βs

Transforming growth factor-βs (TGF-βs) are secreted as latent precursor complexes that are bound to the extracellular matrix (ECM) via latency-associated peptide (LAP) and latent TGF-β-binding protein (LTBP). Only after being separated from these factors (*e.g.* by plasmin, MMP9, thrombospondin-1, integrins and reactive oxygen species, which are found in areas of damage and/or inflammation) can TGF-β bind to its receptor [332, 333]. In this sense TGF-β can be considered a sensor of damage to the ECM [332]. TGF-β is secreted by many cell types, and TGF-β also induces its own expression [333]. TGF-β is thus present from the early stages of wound healing, and is one of the most studied molecular factors in wound healing.

TGF-βs exist in three isoforms in birds and mammals; TGF-β1, -2 and -3, which all have several important, but differing functions in wound healing and development.

During normal development in mice TGF-βs are expressed from early on, and the perinatal death of null mutants for all three TGF-β isotypes underlines the importance of these molecules for normal development [334].

In fetal wounds TGF-β1 expression is lower and resolved faster compared to adult wounds. On the other hand, TGF-β3 expression is increased and prolonged in fetal wounds [21, 333, 335]. TGF-β1 is one of the most potent profibrotic cytokines [265], since it attracts neutrophils, macrophages and fibroblasts to the wound [333], and induces fibroblasts ECM production [20, 333, 336]. It also is responsible for the differentiation of fibroblasts into α-SMA-expressing myofibroblasts in the presence of mechanical stress and the ED-A splice form of FN [170, 286]. TGF-β1 is expressed early at the wound site, but is not able to stimulate myofibroblast differentiation until a sufficiently stiff ECM has being laid down. Conversely, myofibroblasts fail to develop under sufficient mechanical stress in the absence of TGF-β1 [170]. TGF-β1 has also been found to induce leukocyte apoptosis, which is important during transition between the different wound healing phases [83]. In simple *in vitro* settings, TGF-β1 and -3 often have similar effects [337]. However, *in vivo* one of the most successful clinical mediators of TGF-β1 is TGF-β3 [165], although in some experiments TGF-β3 has made no difference on wound healing outcome [333]. In humans, one action of TGF-β3 is to differentially control migration of dermal and epidermal cells. Dermal fibroblast migration is halted with no restriction on epidermal keratinocytes [338, 339]. This supports wound closure whilst limiting fibrosis.

All three isoforms have been described from fish, although only TGF-β1 and -2 from carp [340-343]. TGF-β3 was reported from Siberian sturgeon (*Acipenser baeri*), rainbow trout and European eel (*Anguilla anguilla*) more than a decade ago [342], but the expression of this isoform was not studied in fish until very recently in zebrafish [344]. Here it was found that TGF-β3 expression was upregulated in the wound for longer than the other two isoforms. A fourth TGF-β isotype with high expression levels in muscle and skin was described from gilthead sea bream (*Sparus aurata*) [345] and a second paralog of TGF-β1 was recently described from of rainbow trout [346].

TGF-β1-stimulated *in vitro* fibroblast proliferation is reported from a cyprinid teleost and TGF-β1 thus possibly also stimulates fibrosis in fish as in mammals [347]. A few other studies have investigated the effect of teleost TGF-β1 *in vitro*, and these investigations collectively suggest a

pleiotropic role of TGF-β also in teleosts, as the effect of TGF-β1 is strongly influenced by cell type and activation level [347-351].

The expression of TGF-βs are reported in **Papers II and III**.

2.5.4 Interleukin (IL)-6 and IL-6 family member M17

IL-6 family cytokines include IL-6 and the fish-specific member M17. IL-6 family members share a common signal transducing receptor protein (glycoprotein 130 (gp130)), which results in partially overlapping biological functions [352].

IL-6 is important for several events in wound healing. IL-6 plays an important role in fibroproliferative diseases [265], but also promotes neutrophil apoptosis [353]. IL-6 knock-out mice and wild-type mice injected with anti-IL-6 antibodies show delayed wound healing due to dysregulation of several events including leukocyte infiltration, re-epithelialization, angiogenesis and collagen accumulation [354, 355]. IL-6 and IGFs are involved in differentiation of myogenic progenitors into mature myotubes [155], and IL-6 has also been described as a "myokine" since it is upregulated in working muscle [356]. Rats immobilized following wounding had an accelerated granulation tissue formation, but if kept immobilized beyond 5 days they had excessive wound contraction and scarring [357].

IL-6 has been cloned in rainbow trout [358]. In many respects rainbow trout IL-6 function resembles mammalian IL-6. It is induced in rainbow trout macrophages by the addition of PAMPs (LPS and polyI:C) and IL-1β. In addition, recombinant IL-6 induced STAT3 phosphorylation and expression of SOCS1 and -3, CISH and IRF-1 as in mammals [359]. Treating macrophages with IL-6 had an anti-inflammatory effect by induction of antimicrobial peptides and downregulation of IL-1β and TNF-α [359].

The IL-6 family member M17 shares sequence similarities (albeit not high) to other higher vertebrate IL-6 family members such as ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF). LIF is anti-inflammatory and is upregulated in regenerating skeletal muscle, and knock-down negatively affects regeneration [360]. Orthologs of CNTF or LIF have not been described from teleosts [361].

Rakus et al [362] found M17 to be upregulated following a viral infection, but this was seen only in the carp line R3, and not in the carp line K. Fujiki et al [363] found M17 expression in brain and in activated peritoneal macrophages of carp. A similar expression pattern was found for goldfish M17, and treatment of goldfish macrophages with recombinant M17 stimulated

proliferation and activation [364]. In other teleosts M17 expression is similar, but slightly more skewed towards immune-related cells and tissues, and M17 is induced by PAMPs just as IL-6 [361, 365]. Evidence thus points to an immune function of M17 as well as involvement in the nervous system.

The expression of IL-6 was investigated during rainbow trout wound healing (**Paper III**) and M17 during carp wound healing (**Papers I and II**). See these papers for more information on IL-6 and M17.

2.5.5 Matrix metalloproteinase (MMP) 9 and MMP13

MMPs are zinc-dependent endopeptidases. Two of the MMPs that have been most investigated in the context of injury are MMP9 and MMP13, although the former has received the most attention, also in fish. MMP9 (also called gelatinase B) is a type IV collagenase, which mainly degrades basement membrane collagen [233], and MMP13 (collagenase 3) mainly degrades collagen type II, but also other fibrillar collagens such as type I and III [366].

MMP9 facilitates epidermal migration by detachment from the basement membrane [19, 367, 368]. In fact, epithelial migration seems to be the event mainly affected by MMP-9 *in vivo* [369, 370]. However MMP9 is also involved in initiation of inflammation [233, 371-373], resolution of inflammation [372, 374, 375] and angiogenesis [376].

MMP13 is important for several events during wound healing, including epithelial migration, angiogenesis, granulation tissue formation and wound contraction [369, 370]. MMP13 also induces MMP9 and other MMPs, and MMP13 KO mice have a lower expression of these MMPs after wounding [370].

In humans, MMP9 is expressed mostly by epithelial cells and myeloid cells [233]. However, other cells such as fibroblasts and endothelial cells also produce MMP9 [318, 376-379]. MMP9 expression is induced by TLR activation in human epidermal keratinocytes, and thus by wounding or infection [380]. In fact, MMP9 is one of the genes that are most highly upregulated in wounds [367, 381]. In fetal wound healing MMP9 expression is even higher than in adults [21, 382].

MMP9 and -13 are also important during wound healing in fish [4, 367, 383-385]. In salmonids infected with sea louse (ectoparasites which essentially inflict excisional dermal wounds) MMP9 and MMP13 are differentially regulated [4, 386]. In carp, MMP9 seems to be involved in inflammation and remodeling phases [385].

In adult teleosts MMP9 expression is highest in leukocyte-rich organs [385, 387, 388]. When comparing acidophilic granulocyte and macrophage MMP expression in gilthead seabream Castillo-Briceño et al [387] found that macrophages have a higher expression of MMP2 (c. 100-fold), similar expression of MMP9 and lower expression of MMP13 (c. 10-fold). However, in fat snook (*Centropomus paralellus*), neutrophils were the only peripheral blood leukocytes positive for MMP9 [188]. These studies could thus indicate that MMP9 expression in fish is also mainly restricted to myeloid cells, although to different types in different species. However, in zebrafish fin regeneration studies expression of MMP9 induced at the wound edge is mostly from cells that are not of myeloid origin [383, 388].

In this thesis MMP9 expression is investigated in carp wound healing and development in **Paper II** and MMP9 as well as MMP13 expression during rainbow trout wound healing in **Paper III**.

2.5.6 CD163

CD163 is a membrane scavenger receptor of the cysteine-rich scavenger receptor super family type B. It is a receptor for haemoglobin-haptoglobin complexes and is thus involved in the clearance of haemoglobin, and this interaction triggers expression of anti-inflammatory IL-10 [389, 390]. In addition, CD163 has been found to interact with bacteria, in which case the pro-inflammatory cytokine TNF-α is produced [389].

Its expression is restricted to circulating monocytes and macrophage subsets, with a higher expression in mature tissue macrophages than in monocytes [389, 391]. Infiltrating macrophages are CD163-negative [389]. Macrophages expressing high levels of CD163 are the predominant macrophages during the resolution phase of inflammation, and CD163 expression is stimulated by anti-inflammatory mediators such as IL-10 and glucocorticoids, but also by the more pleiotropic IL-6 [389, 392].

A CD163 homolog is also present in the genome of several fish species, but curiously apparently not in birds and amphibians [393]. However, CD163 has not been coupled to macrophage polarization in fish, but CD163 wound healing expression patterns are consistent with such a link in rainbow trout (**Paper III**).

2.5.7 Tenascin-C (TN-C) and fibronectin (FN)

Fibronectin (FN) is a glycoprotein dimer produced in a soluble or insoluble form. The former is produced by the liver and constitutes a significant fraction of plasma proteins. This type of FN is initially deposited from plasma after injury, and is replaced by insoluble FN, which is initially produced at the wound site by macrophages and later by fibroblasts and myofibroblasts [168, 394].

FN guides fibroblasts and inflammatory cells to the wound [168], and many growth factors that participate in the differentiation of fibroblasts into myofibroblasts are bound to FN [282]. FN is a necessary prerequisite for collagen deposition [395], and FN fragments are thought to be involved in wound contraction through their interaction with $\alpha_4\beta_1$-integrins [396].

Tenascin-C (TN-C) is a glycoprotein structurally similar to FN. During homeostatic conditions TN-C is expressed at the dermal-epidermal border and in larger blood vessels. It is induced already early in wound healing and TN-C promotes epithelial cell migration and proliferation [397]. IL-1β has recently been shown to stimulate fibroblasts to produce TN-C [398]. TN-C and especially FN form a major part of the granulation tissue, but they play somewhat different roles as full-length TN-C antagonizes the cell adhesive effect of FN and stimulates fibroblast migration [20]. However, degraded TN-C fragments inhibit migration [399]. The granulation tissue is rich in newly forming blood vessel, and the high expression of TN-C in granulation tissue seems to stem specifically from the endothelial cells of sprouting vessels [397].

TN-C is involved in several stages of wound healing, and has a highly pleiotropic role. TN-C induces inflammation when injected into mice, and in vitro studies show that macrophages and fibroblasts are stimulated to produce pro-inflammatory cytokines by TN-C [397]. TN-C *null* mice have a faster resolution of inflammation, a lower collagen and FN expression [168, 397] and generally do not exhibit fibrosis [282]. On the other hand, in some aspects TN-C seems to favour a Th2 environment as it has an inhibitory effect on monocyte chemotaxis, but stimulates lymphocyte chemotaxis, and additionally lymphocytes are stimulated to produce the Th2 cytokines IL-4, IL-5 and IL-13, although also IFN-γ [397, 400].

TN-C is upregulated from a couple of days and onwards in the regenerating limb of the salamander. It is specifically expressed in the blastema, where it provides important instructive cues to satellite cells and dedifferentiating skeletal muscle by stimulating myoblast migration and myotube fragmentation. On the other hand, FN stimulates fusion of myoblasts and is downregulated in the blastema [31]. Compared to mammals axolotl full-thickness excisional skin wounds a relatively long delay in production of new ECM, and this consists of less FN and more TN-C than in mammals [37].

TN-C and FN expression was investigation in relation to wounding in rainbow trout (**Paper III**).

2.5.8 Collagen type I

At least 27 types of collagen are found in vertebrates. Collagen type I is the main fibrillar collagen found in muscles, and is structurally important for the transmission of muscle contraction forces [303].

Collagen III mainly produced during the early proliferative phase, is later replaced by the stronger and stiffer collagen I during late proliferative and remodelling phases [401].

Fibroblast and especially myofibroblasts are the main cell types responsible for the production of ECM molecules, including collagens. In mammals, collagen production is initiated about 3-5 days after injury by growth factors such as TGF-β, PDGF, FGF-2 and IGF-1 mainly secreted by macrophages [151].

Collagen has not been much studied in fish in the context of wound healing, but in gilthead seabream collagen fragments were able to stimulate production of IL-1β and MMP9 and -13 in acidophilic granulocytes, macrophages and fibroblasts *in vitro* [288, 402].

Collagen type I α1-chain expression was investigated in wounded rainbow trout (**Paper III**).

2.5.9 Prolyl 4-hydroxylase (P4H)

Secretion of collagen (and a few other matrix molecules such as elastin) from fibroblasts into the ECM only occurs when in the triple-helical form. Hydroxylation of certain proline residues of collagen pro-α-chains is essential for this triple helix formation, and the principal enzyme involved in this process is prolyl 4-hydroxylase (P4H) [403].

There are several forms of prolyl 4-hydroxylases (P4Hs). Apart from the P4Hs involved in collagen production (C-P4Hs), two other P4H families are found in vertebrates, but these do not contribute to collagen triple helix production. Three forms of C-P4Hs are found in humans depending on their use of α-chain. C-P4Hs are tetramers with two common β_2-subunits and two identical subunits of either α_1, α_2, or α_3, and the resulting tetramers are called C-P4H-I, -II and –III, respectively [404]. C-P4H-I is the most common form in most human cell types and tissues, and it is the expression of the α_1-subunit that is investigated in **Paper III**. C-P4H-II is expressed mostly in chondrocytes [405] and C-P4H-III at much lower levels than the other two types anywhere. C-P4H activity is increased in several fibrotic disorders [404].

2.5.10 Lysyl oxidase (LOX)

The main function of LOX is oxidation of lysine residues in collagens and elastins to form spontaneously reacting peptidyl lysines. This facilitates cross-linking of these matrix molecules, which provides rigidity and tensile strength to the matrix [406].

Since the mechanical properties of the matrix influences the cells within it, LOX activity is an important regulator of wound healing processes. LOX is upregulated during tissue repair and fibrosis [407], and it is induced by TGF-β [408]. In rat, LOX expression in the skin peaked 3 days after wounding, which was prior to a rise in expression of collagen type III, a fibrillar collagen often produced in wounds and later replaced by collagen type I [409]. Conversely, LOX expression is downregulated in fetal scarless wound healing [410]. LOX is essential for normal muscle function, and LOX *null* mice die perinatally [406]. LOX is also important for keratinocyte differentiation and maintenance of epidermal homeostasis [411].

LOX expression has not been previously studied in fish, but the hydroxylysyl pyridinoline cross-links formed by its action has been studied in Atlantic salmon, where these have been shown to have a strong influence on texture [412, 413].

Neither P4H nor LOX expression has been previously studied in fish. **Paper III** describes the expression of these enzymes during wound healing in rainbow trout.

2.5.11 Heat shock protein (HSP) 70

HSPs were originally discovered in *Drosophila* following acute heat shock, but have since been implicated in a wide range of stressful conditions. They function as molecular chaperones, which aid in folding and transport of proteins [414], and as such they participate in the initiation of adaptive immune response by chaperoning peptide antigens [206]. HSP70 is considered an inducible HSP with no or limited constitutive expression [415].

HSP70 is also stress inducible in fish. Consequently, most studies on teleost HSPs have focused on their involvement during temperature stress, but also during osmotic stress and in the presence of environmental stressors such as Cadmium and Copper. A number of studies have looked at the involvement of HSPs in wound healing and during ontogeny in fish [414]. These studies generally report constitutive expression of HSP70, but levels vary with species, cell type and tissue as well as developmental stage [414, 416-422].

HSPs have previously been considered DAMPs, but recently this view has been challenged [114, 206], partly because they are not only intracellular but are also actively released. Contrary to

the effects of (other) DAMPs HSPs are usually reported to have anti-inflammatory or homeostatic properties [114, 118, 423-426], and in humans, thermal pre-conditioning is used prior to surgery to dampen inflammation and enhance healing of the surgical wound through an induction of HSP70 expression [427, 428].

In vitro, extracellular HSP70 inhibits TGF-β signaling through interaction with its receptors [429]. Subcutaneous injection with exogenous HSP70 accelerates wound healing in mammals, and this effect is partially through upregulation of macrophage-mediated phagocytosis [430]. HSP70 at the wound edge mostly derives from epidermal and myeloid cells [431, 432].

HSP70 expression during ontogeny in wounded carp is followed in **Paper II**.

2.5.12 Complement factor C3

The complement system is a complex system of secreted and membrane-associated proteins in which complement component 3 (C3) takes a central role. It is mostly regarded as an important part of innate immunity. However, complement has a range of functions, and the complement cascade is also activated after injury [84] and is important for regeneration in urodele amphibians [433, 434]. C3a (a cleavage product of C3) attracts neutrophils and macrophages and stimulates histamine and leukotriene (C4 and D4) release from mast cells [84]. Another effector function of complement is clearing of apoptotic cells and debris by C3a thus avoiding release of danger signals and reducing inflammation [229, 435-437]. Complement thus participates in initiation as well as resolution of inflammation.

The teleost complement system has received great deal of interest, and in particular in species within the cyprinid family [437]. While mammals are reported to possess only one isoform of C3, common carp has five [438] and other fish also have more than one [439-443]. Of the five carp C3 isoforms one is probably non-functional and the other four differ in expression levels and hemolytic activity [444]. Another intriguing discovery is that all five carp C3 isoforms are more similar to each other than C3 from other species. The implications of this have yet to be elucidated [441], and carp C3 has not been investigated in relation to wound healing.

C3 expression was investigated in **Paper II**.

2.5.13 Innate antibodies

Although antibodies are normally considered part of the adaptive immune system, the so-called natural or innate antibodies have a broad binding spectrum and low affinity and are more innate-like. In mammals they are produced by B-1 cells very early in development [445].

Immunoglobulins are not usually directly implicated in wound healing of non-infected wounds, but innate antibodies also bind self-antigens, and are involved in removal of aberrant and apoptotic cells [252, 445, 446] also during wound healing [248]. In fish, innate antibodies are likely to be of particular importance compared to higher vertebrates since teleosts respond relatively poorly to immunization, resulting in low affinity antibodies with limited affinity maturation and a poor secondary response [257-261, 268, 447], and it appears that the poorer the response, the higher the titers of innate antibodies [445, 448, 449]. Additional hints for B1 properties of piscine B-cells comes from the fact that innate mammalian antibodies are mainly IgM [251, 445], which is the major immunoglobulin in fish and the fact that antigen-specific responses and persistent memory of B1 cells can occur in the absence of germinal centers, which are primitive in teleosts [268, 450-453].

In teleost fish, three immunoglobulin classes are recognized; IgM, IgD and IgT/IgZ [449]. There is no class switch in fish and IgT^+ and IgM^+ B lymphocytes form separate lineages [268]. IgT and IgZ were discovered simultaneously in rainbow trout and zebrafish and represent the same isotype despite the different name. The channel catfish (*Ictalurus punctatus*) is the only teleost investigated thus far to lack IgT, and the coelacanth (*Latimeria chalumnae*) the only fish to lack IgM [454]. On the other hand subclasses exist in some fish. Carp have two subclasses of IgZ, namely IgZ1 and IgZ2 [455]. IgM and IgT/IgZ are usually described as systemic and mucosal immunoglobulins, respectively [456], while the function of IgD is still largely unknown [457, 458]. IgZ1 is more similar than IgZ2 to the prototypic teleost IgT with regards to gene structure, but nonetheless is speculated by Ryo et al. to have a systemic role while IgZ2 is more mucosal [455]. However, Przybylska [459] found a high induction of IgZ1 expression in the skin of adult carp following intravenous injection with β-glucan.

It was an interesting and new observation that the expression of the immunoglobulin IgZ1 was upregulated following wounding in carp larvae (discussed in **Paper II**). In this study we investigated the local gene expression by removing head and viscera prior to RNA extraction. A few fish were processed whole, but were not included in the article since they were too few for a reliable statistical analysis. However, they do support the finding that IgZ1 is upregulated as a result of the wounding and are presented in figure 4. The only significant wound-induced difference in gene expression was found 3 days post-wounding in larvae wounded 7 days post-fertilization for IgZ1. On this particular day two wounded larvae were sampled whole and these

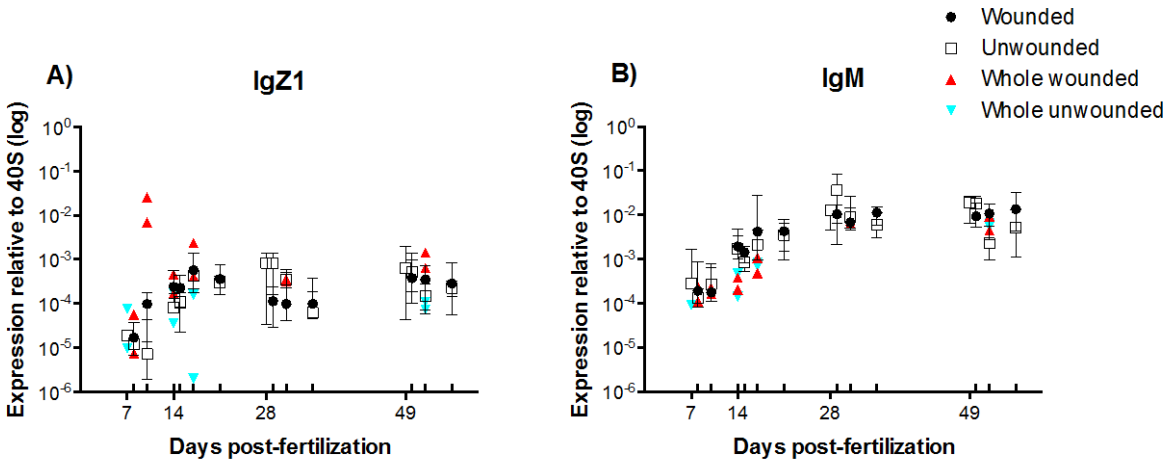


Figure 4. Expression of the immunoglobulin genes IgZ1 (A) and IgM (B) in carp larvae and juveniles wounded 7, 14, 28 and 49 days post-fertilization and sampled 1, 3 and 7 days post-wounding. Black closed circles and open squares represent local responses (head and viscera removed) while red and blue triangles represent gene expression from whole fish. For further description and discussion see **Paper II**.

had a very high IgZ1 expression. Whole larvae from wounded as well as control fish were sampled on day 3 post-wounding for larvae wounded 14 days post-fertilization and juveniles wounded 49 days post-fertilization. On both days IgZ1 expression was higher for the wounded fish. Another interesting result is that Ig transcript levels are comparable in whole fish and fish in which primary lymphoid organs are removed before RNA extraction. This indicates that B lymphocytes are found in significant numbers in the periphery already from the earliest life stages. And in the case of IgM there is even a tendency (although based on very few samples) for IgM expression to be higher in the periphery than in primary lymphoid organs at the late larval stages.

2.6 Pain

A relevant question when conducting potentially painful experimental procedures on fish, or any animal for that matter, is whether it experiences a sensation of pain. Unfortunately, the answer to this question is a matter of continued debate [460, 461].

The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage".

Establishing whether or not fish feel pain is thus difficult due to the cognitive and subjective nature of pain. Since fish are animals that we are not able to communicate meaningfully with this matter can only be approached by: 1) Investigating whether fish have the apparatus necessary to perceive pain, and; 2) identify behaviour that can be interpreted as associated with pain as it has been done in the more popular experimental mammals such as mouse, rat and rabbit [462].

It is clear that fish respond to noxious stimuli [461]. A-γ nociceptors are relatively common in teleosts, but in humans these are more linked with avoidance responses than pain. C type nociceptors are responsible for severe pain in humans, and while these receptors are present in teleosts, they are rare. In addition, homologs of the cortical regions of the brain responsible for pain perception in humans are not found in fish [460], although anatomical homology is a poor indicator of functional homology in comparative studies on the brain (professor Jesper Mogensen, pers. comm.).

There have been several investigations identifying behavioural changes in fish following stimulation with potentially painful substances, such as injection with acid or bee venom, or by infliction of surgical wounds [461]. These behavioural changes involve a rocking motion while resting on the bottom of the aquarium and rubbing of the affected area. Cyprinids and salmonids are among the most investigated species in this respect.

During my studies I have not systematically made behavioural observations. However, during my daily routines in the fish-keeping facilities (feeding fish, changing water etc) I have not noticed any behavioural difference between wounded and control fish.

The use of analgesics in fish research is not common practice. However, this is not due to a lack of responsiveness to analgesics. Two basic forms of analgesics are used; opiates and non-sterical anti-inflammatory drugs (NSAIDs). Fish have μ and k receptors for opiates, thus making it reasonable to expect an effect of opiates. Fish also have the COX enzymes that NSAIDs inhibit [461].

Harms et al [463] investigated the effect of the analgesics butorphanol (an opiate) and ketoprofen (an NSAID) on post-operative behaviour and clinical pathological changes of Koi carp. They found a positive effect of butorphanol on the behaviour of post-operative carp, with less signs of putative pain such as reduced activity and feeding. In addition, intra-operative administration of ketoprofen led to reduced muscle damage – likely through the anti-inflammatory property of this drug.

Thus in relation to wound healing studies the use of NSAIDs would likely have an undesirable influence on the healing process. Opiates are probably a better alternative. However, MS-222 (a sodium channel blocker) also has analgesic properties, although it is more commonly used on fish due to its anaesthetic effects [461]. MS-222-induced anaesthesia may itself indirectly affect wound healing, since it can lead to increased stress hormone and lactate levels in fish [461]. Stress as well as corticosteroids administered after wounding impair wound healing in mammals by leading to reduced inflammation, collagen production and contraction [84, 464]. On the other hand, lactate is known to stimulate VEGF production (and thus angiogenesis) and collagen production by fibroblasts [465].

2.7 The wound healing model

2.7.1 The investigated species

Cyprinids and salmonids are among the most investigated teleost taxa, also regarding wound healing. Common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) are large representatives for the two taxa respectively. They are both important species in aquaculture, and many ESTs and annotated genes are available in GenBank for both species. In addition, common carp is a close relative of the zebrafish, a model organism in vertebrate wound healing the genome of which is completely sequenced. Thus these species were appropriate for gene expression studies. Carp had the added advantage over rainbow trout to be available in a domesticated strain called mirror carp, which has few, scattered scales. The scaleless areas are ideal for infliction of standardized wounds, since it eliminates the need for prior removal of scales and thus disrupting the epidermis in a larger area than the actual wound site. Table 1 (see section 2) lists some of the characteristics of the two species.

2.7.2 Biopsy punch wounds

The biopsy punch (fig. 5) is a standard tool for wound healing studies, since it is easy to use and creates wounds of a completely standardized size. The progression of healing of the excisional wounds created this way is easy to monitor continuously by different macroscopic imaging techniques *in vivo* (fig. 6). In addition sampling for gene expression studies can be equally standardized by use of larger biopsy punches (see **Paper I** for an illustration).

Figure 5. A biopsy punch.

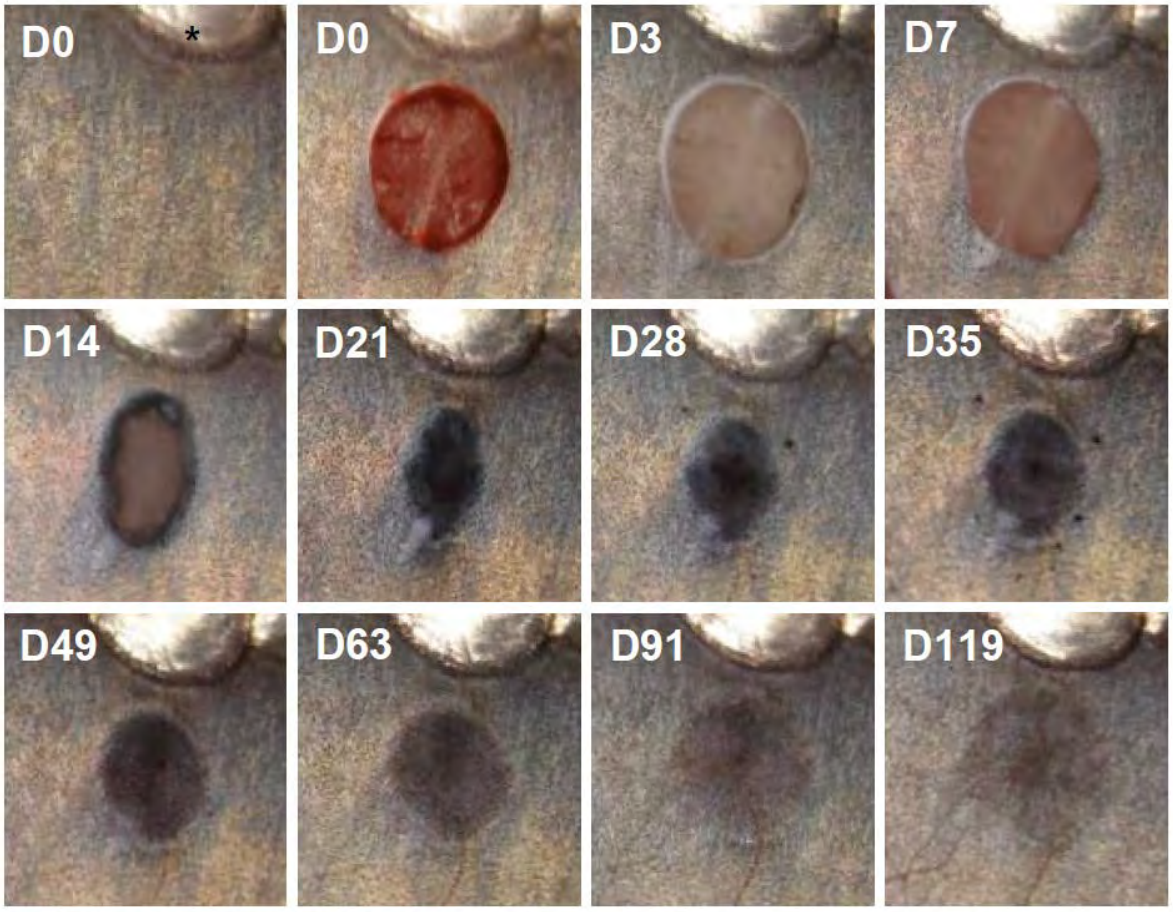


Figure 6. Healing of a full-thickness cutaneous 6mm biopsy punch wound in a common carp. D is day after wounding. A scale is seen in the top of the images, and is marked with an asterisk on the top left image. Notice the initial retraction and lightening of the wound edges on day 3. The lightening of the wound edge is probably a result of epidermal cells having migrated over the wound bed and thus exposed the lighter dermis at the edges. The wound edge starts to darken after about a week, and at the same time the wound starts contracting. In this example the wound is fully closed and contracted at day 21 and the wound area is now very dark. From now on the hyperpigmented area expands and fades. This could be due to myofibroblast apoptosis, and thus release of the contractile forces in the wound. Spots appear at day 28. These are most often found in the periphery of the wound and are possible melanomacrophage centers (see Discussion). They often start forming around three weeks post-wounding and persist for a few weeks. Blood vessels appear in the skin around the wound area at the late stages of wound healing. In this case external visual progression of wound healing was monitored until day 119 after wounding.

2.7.3 Gene expression

Since availability of antibodies and other molecular tools for studying rainbow trout and carp are limited, but many nucleotide sequences are available, gene expression is arguably the best method of studying wound healing at the molecular level in the chosen species.

Gene expression studies with carp and the fish louse *Argulus* have indicated that fish skin may in some respects act as a well-defined organ since gene expression in infected and non-infected areas of infected fish have similar changes in gene expression compared to non-infected fish [466]. To test if the same could the case with muscle tissue, we included internal (a non-wounded site in a wounded fish) as well as external (non-wounded fish) control samples in one of the studies (**Paper III**).

A pitfall with gene expression analysis in fish, and especially salmonids, is the presence of several isoforms and/or pseudogenes following one (two in salmonids) whole-genome duplication events in the teleost lineage [74]. These duplicate genes may be redundant, or have evolved to perform different functions.

2.7.4 Image acquisition and analysis

One of the objectives of the study was to test different imaging systems on the healing wounds. The classical imaging technique used on healing wounds is conventional light, confocal, transmission electron or scanning electron microscopic images of histological sections. However, in recent years new imaging techniques such as two-photon imaging combined with biological models (*e.g.* transgenic zebrafish) have brought new insight into *in vivo* cell migration and communication [70, 467]. However, creating transgenic carp and trout were outside the scope of this study.

Multi- and hyperspectral imaging

We instead tested two related imaging techniques: Multispectral and hyperspectral imaging. These imaging techniques both result in a number of grayscale images that each represents a narrow range of wavelengths. In the case of the multispectral images we used a VideometerLab (Videometer A/S, Hørshom, Denmark). This imaging and lighting device is equipped with 20 different diodes each with a narrow emission range within the electromagnetic spectrum. Each diode strobes sequentially and an image is acquired each time. The 20 individual images cover the range from ultraviolet (UV, 375nm) over the visual spectrum to near-infrared (NIR, 1050nm). The longer wavelengths penetrate deeper into the tissue than the shorter, thus each of the 20 images provide different information of the wound area (see Fig. 7 for samples). A drawback to this technique is the large image files created (the image cube has the dimensions of 1280x960x20 pixels), and the lack of specific software to analyze them, which makes the image analysis time consuming. We instead decided to shift to ordinary digital RGB images and an imaging set-up with uniform lighting.

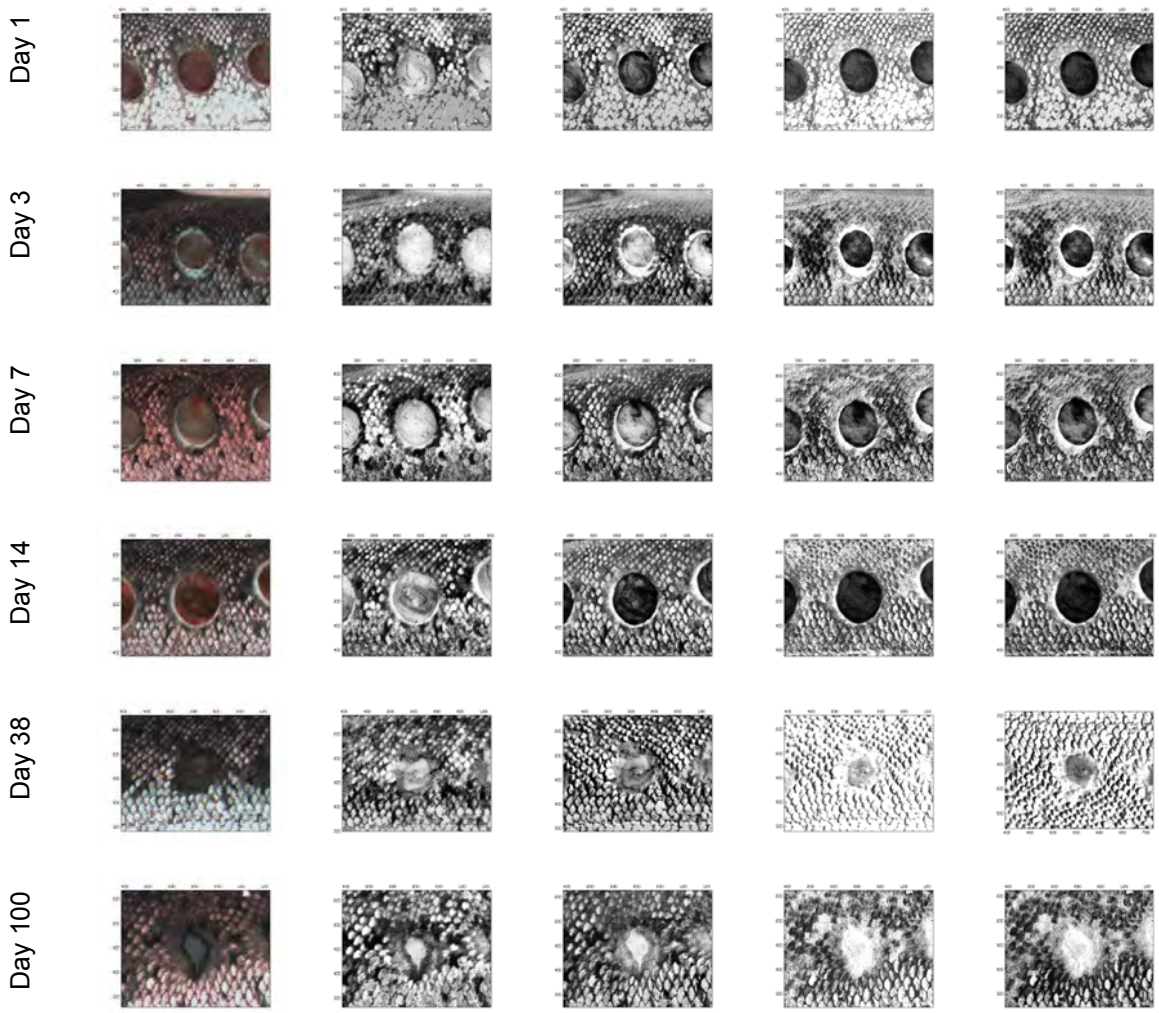


Figure 7. VideometerLab images of healing wounds in rainbow trout (Paper III). The images are samples that show the different features that can potentially be discerned with this imaging technique. Each row represents a random fish from each sampling day (days 1, 3, 7, 14, 38 and 100 after wounding). The left column shows pseudo RGB image representations of the grayscale images. Each of the other columns are different combinations of selected wavelengths.

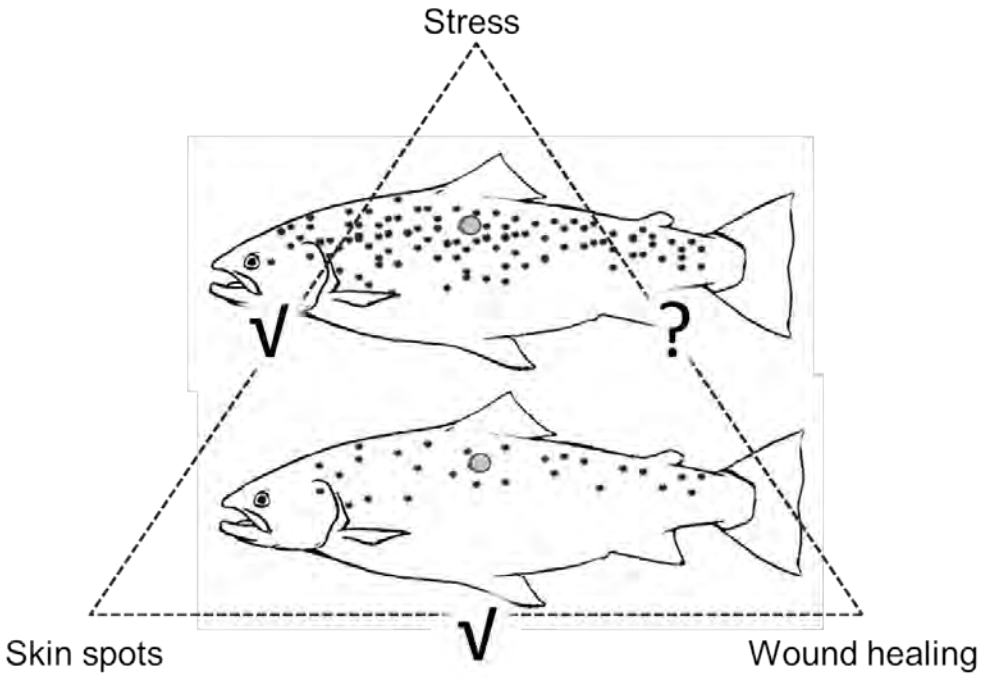
Wound closure

We used the multispectral as well as ordinary RGB digital images to estimate wound closure. For the purpose of the studies included in this thesis we did not consider re-epithelialization wound closure. Instead we measured the open wound area as the area inside the dermal wound edge. This was very easy to demarcate accurately at early wound healing stages. However, as the wound healing progressed and the wound edge became hyperpigmented it was increasingly difficult to define. In addition, the intensity of the hyperpigmented wound edge was individually variable. At our hands wound closure is thus likely a combination of contraction and thickening of the epidermis at the wound edges, and a reconstruction of dermis. We created MatLab scripts

to automatically calculate the open wound area, but wound edge detection failed at late healing stages due to the variable nature of the appearances of the wounds. We instead traced the wound edge manually and imported these into MatLab where the wound area was measured.

3. Unpublished results

3.1 Number of melanin-based skin spots negatively correlates with wound closure in rainbow trout (*Oncorhynchus mykiss*) – an image analysis study



Graphical illustration of the relationship of stress, number of skin spots and speed of wound healing. A relationship between the number of melanin-based skin spots and the stress response has been demonstrated previously. Here we demonstrate that number of skin spots correlate with wound healing. However, a definitive connection between wound healing and stress response could not be made.

Introduction

Physiological stress (elevated corticosteroid levels) is negatively correlated with wound contraction and overall wound healing in mammals [464, 468, 469]. One of the most recognized effects of corticosteroids is the attenuation of acute inflammation with a reduction in production of proinflammatory molecules such as TNF-α, IL-1β and IL-8, and also MMP9, in the wound [464, 470]. Nonetheless, inflammation in mammals is usually associated with accelerated wound healing, but at the expense of scarring [153].

Inflammation has a limited influence on wound healing, at least in zebrafish [143], but how stress influenced wound healing has not been directly addressed in fish. However, recently Krasnov et al [3] studied the effect of intraperitoneal cortisol injection on skin gene expression

of sea lice-infected Atlantic salmon. Sea lice are ectoparasitic crustaceans that feed on the skin and in severe cases also muscle, and thus induce chronic ulcers. Skin was sampled 20 days after cortisol injection (18 days after infection with sea lice). Transcription of inflammatory cytokines and extracellular matrix molecules were downregulated, and the authors conclude that the gene expression profile was consistent with compromised wound healing in cortisol-injected salmon when comparing to mammals. However, MMP9 and MMP13 were upregulated in the skin of cortisol-treated salmon, and these are important for wound healing *e.g.* by facilitating epidermal migration and wound contraction [369, 370]. In zebrafish Mathew and co-workers [143] found that corticosteroids had no apparent effect on inflammation, but that they did adversely affect blastema formation and thus healing of amputated caudal fins.

Kittilsen and co-workers [471] noticed that two rainbow trout strains that had been selected for high and low cortisol response to stress through four generations differed with respect to the number of melanin-based skin spots. Salmonids show a large degree of plasticity of the number, size and pattern of skin spots. This skin spot pattern is relatively fixed in each individual fish, and the number of skin spots shows a strong heritability and a limited environmental influence [472]. It was further speculated by Kittilsen and co-workers [471] whether the spots were a manifestation of the coupling of the hypothalamus-pituitary-interrenal (HPI)-axis with melanin-based pigmentation. To test if this divergent spot pattern was just an artefact of the selection for stress response, or if it was indeed correlated to the stress response, a non-selected population of another salmonid Atlantic salmon (*Salmo salar*) was divided into two groups: One group contained fish with few spots, and one group contained fish with many spots. These two groups were used to test the stress response by observing behavioural and physiological traits. This experiment demonstrated that the group with many spots had lower post-stress levels of cortisol and had a higher feed intake after stress [471]. This correlation with stress response and feed intake is well in line with previous studies in rainbow trout in which low cortisol levels were associated with social dominance [473].

Given this correlation of fish skin spots><stress response, and mammalian stress><wound healing, we tested whether number of skin spots in rainbow trout correlated with wound healing by measuring wound closure kinetics.

Materials and methods

Experimental set-up

Rainbow trout (size at start of experiment: 109.5±15.5g, 20.8±1.2cm) were sorted into five 250L aquaria, 25 fish in each. The aquaria were fitted with Eheim classic filter pumps and airstones (Eheim, Germany). In addition, half the water was substituted with fresh, temperature-adjusted, oxygenated tap water daily. The temperature was kept at 15±1°C, and the light cycle was 12:12h light:dark.

Figure 3.1.1. The experimental facilities at DTU FOOD where the fish were kept during the course of the experiment.

The fish were kept in the experimental facilities for 14 days prior to commencing the experiment. The fish had been kept on minimal fed until being moved to the experimental facilities. Here they were fed 1.3% initial body weight daily from day 14 prior to the start of the experiment and throughout the experimental period. Clock automatic feeders were used to spread the feeding over a course of several hours.

On the day of starting the experiment, the fish were anaesthetized in 100mg/L MS-222 one by one, and a cylinder of tissue was excised with an 8mm Ø biopsy punch (Medinor, Oslo, Norway) on the left side, thus creating a wound with a diameter of 8mm and a depth of approximately 2mm. The wound was made in an area just above the lateral line, below the dorsal fin. A digital image was acquired using a special box with uniform lighting and a digital Canon SLR camera. The fish was then measured and weighed before being released back into the aquarium.

On days 14, 21 and 28 post-wounding, the fish were once again anaesthetized before a digital image was acquired. However, on day 28 the experiment was terminated and the fish were killed in an overdose of MS-222 and weighed and measured.

The wound edges on the digital images were outlined manually, BLOBs (Binary Large OBjects) corresponding to size of the wound openings were created with Adobe Photoshop and the relative size of the BLOBs was determined using MatLab (fig. 3.1.2).

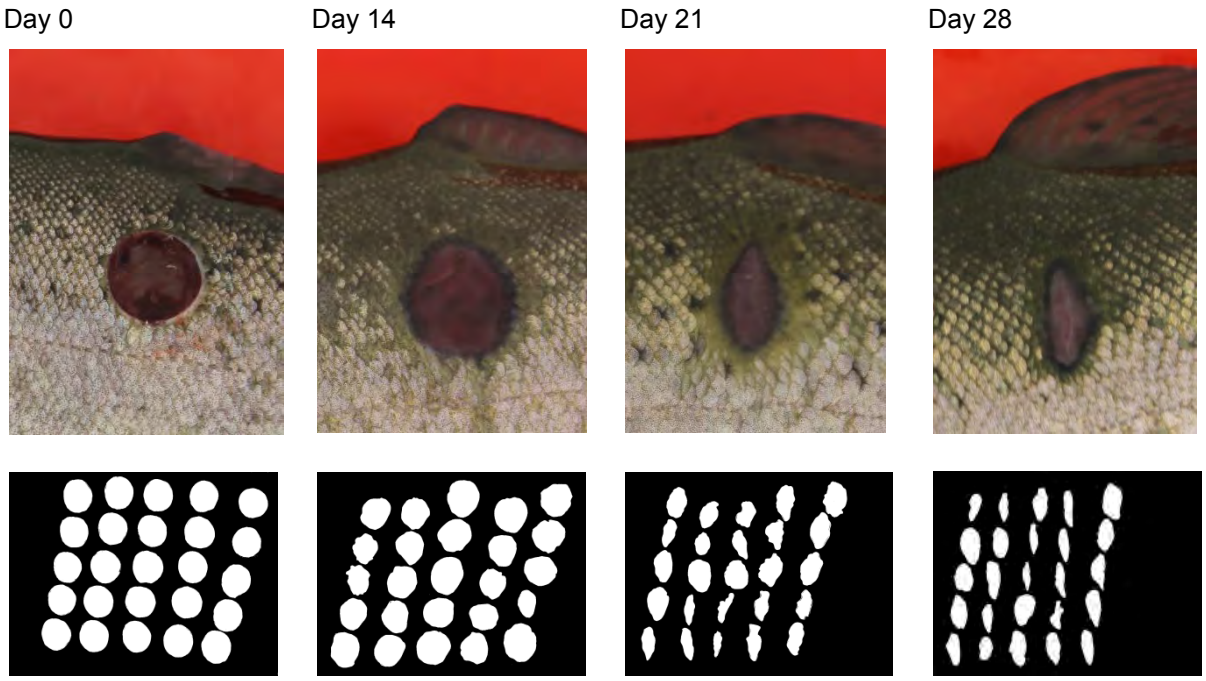


Figure 3.1.2. Digital images and BLOBs. Top row: Examples of the digital RGB images of fish on each of the days 0, 14, 21 and 28 after wounding. Bottom row: BLOBs from 25 fish from one of the aquaria.

Data analysis

Each digital RGB image was imported into MatLab. In simple terms, the MatLab workflow started with rotating the image. Then the wound and lateral line was identified, and the image was divided into four quadrants by a vertical line running through the center of the wound and

a horizontal line starting at the lateral line just below the wound. The spots were then counted in the upper left quadrant (Fig. 3.1.3).

Figure 3.1.3. Sample images of stages in the spot detection. To the left is an example of a rainbow trout with few spots, and to the right an example of a rainbow trout with many spots. The fish are from day 28. The blue cross on the lower images represents the two lines running through the center of the wound and along the lateral line. Each red circle represents a detected skin spot. The spot detection is not perfect. Especially on the dorsal part of the fish where there is less contrast between the background and the spots, these are not detected.

The correlation of wound size and number of skin spots was tested with Pearson's *r* due to the apparent linear and elliptical distribution pattern (Spearman's rank test was also tested with similar results).

Results

There was an increasingly positive correlation between wound size and number of skin spots from day 0 to day 28 after wounding, which was only significant at day 28 (Table 3.1.1 and figure 3.1.4).

	Pearson's correlation	P-value
Day 0	0.058	0.527
Day 14	0.094	0.301
Day 21	0.145	0.111
Day 28	0.201	0.029*

Table 3.1.1. Pearson's correlation and P-values for wound size and number of skin spots. The asterisk marks a statistically significantly positive Pearson's correlation at day 28 after wounding.

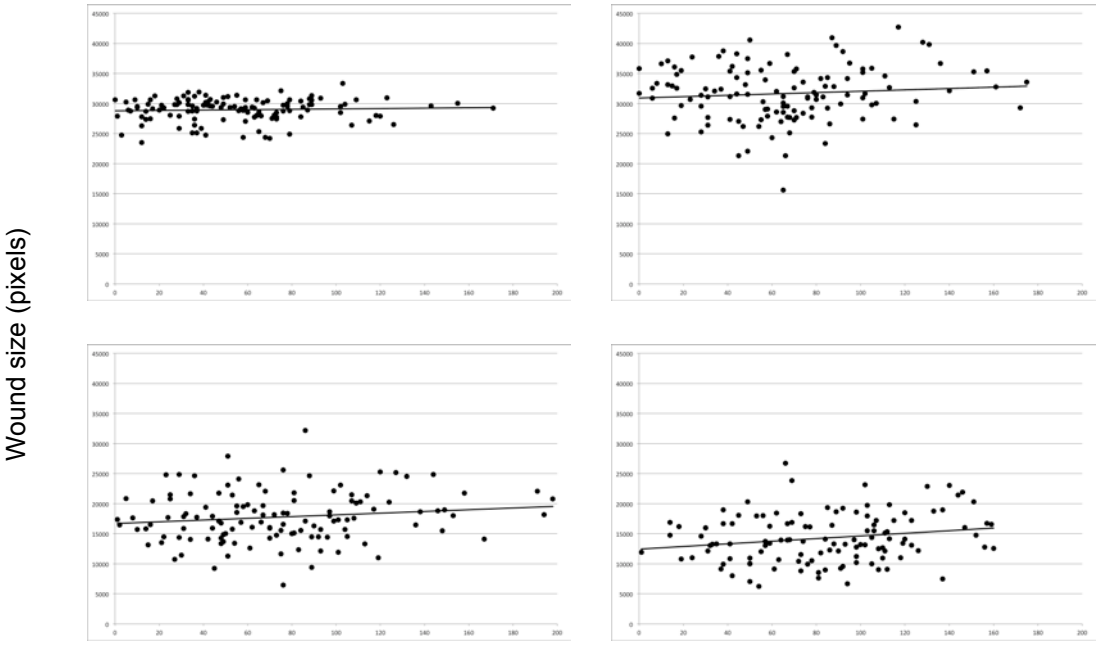


Figure 3.1.4. XY plots of wound size and number of skin spots with trend line at days 0, 14, 21 and 28 after wounding. There is an increasingly positive Pearson's correlation from day 0 to day 28, which is significant at day 28.

Discussion

The influence of skin spots on wound healing was small but significant. Contrary to what could be expected based on the mammalian wound healing response to stress the fish with more spots (and thus a lower stress response, although this was only circumstantial evidence) were slower healers.

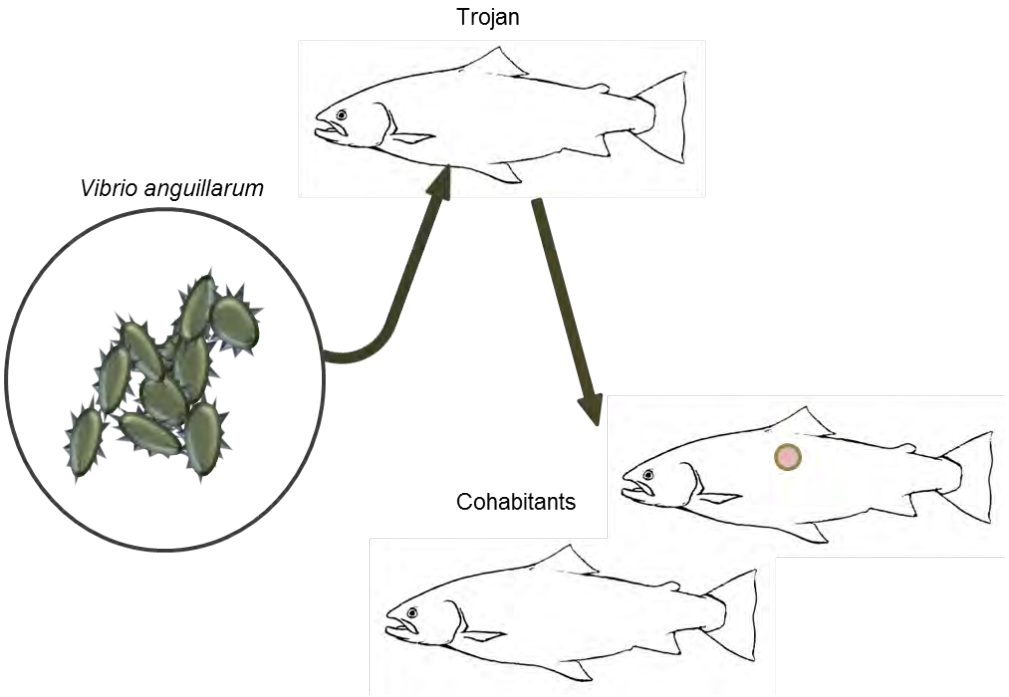
In mammals, melanins are synthesized by melanocytes, which are pigment cells derived from the neural crest mesenchyme [8]. Fish melanocytes are also melanin-producing. In addition to melanocytes lower vertebrate macrophages (melanomacrophages) also produce melanin, and these are not derived from the neural tube [8].

The melanization of fish skin wounds appears to be due to the presence of melanocytes [45, 61], but the abnormal presence of pigments in salmon visceral organs and musculature results from aggregates of melanomacrophages [8].

Tyrosinase (as well as 5,6-dihydroxyindole-2-carboxylic acid (DHICA) oxidase (TRP1) and dopachrome tautomerase (TRP2)) is involved in the melanogenic pathway, and the Atlantic salmon macrophage cell line (SKH-1) expresses tyrosinase, TRP1 and TRP2 [8]. In this cell line the tyrosinase gene is highly upregulated at 20°C, there is some expression at 15°C, but none at 10°C [474]. Since 20°C is stressful for a cold-water species such as the Atlantic salmon, it appears that melanin is not directly involved in immunity, but is a powerful antioxidant that protects cells during stress [474].

It could thus be interesting to further investigate the involvement of these two melanin-producing cell types in fish inflammatory responses in full-thickness cutaneous wounds. To this end, transparent transgenic zebrafish such as pinky (pk) may be useful. Pk was designed to visually study cell migration *in vivo* [47], and has a dramatic reduction in chromatophore numbers.

3.2 Effect of the fish pathogen *Vibrio anguillarum* on wound healing in rainbow trout



Graphical illustration of the cohabitation experiment. Trojans were infected with *Vibrio anguillarum* intraperitoneally. They were released into a tank with non-injected cohabitant rainbow trout with or without wounds. The cohabitants were exposed to bacteria deriving from the Trojans.

- Mortality was not affected by wounding, regardless of bacterial challenge.
- Scratch-wounded fish may function as secondary seeder fish.
- Wound closure was not significantly affected by bacterial challenge.
- Challenge-related differences in gene expression were most prominent at day 12, when expression of IGF-1 and collagen type I was higher in challenged fish.
- IGF-1 was downregulated and FGF-2 was upregulated in wounds on day 12.

Introduction

We used a cohabitation model of infection, where rainbow trout (*Oncorhynchus mykiss*) were intraperitoneally injected with *Vibrio anguillarum*. These so-called Trojan fish subsequently infected the cohabitants in the tank. We inflicted two kinds of wounds prior to challenge; superficial wounds by scraping or deep wounds created with a biopsy punch.

The following month we registered mortality daily and healing of punch wounds at day 3, 12 and 29 after wounding. On days 12 and 29 we additionally measured the combined effect of cohabitation and wounding on expression of Collagen type I α1 chain (Col1a1), fibroblast growth factor 2 (FGF-2) and insulin-like growth factor 1 (IGF-1) in the healing wound.

FGF-2 is important for granulation tissue formation following wounding in mammals. The importance of FGF signaling for granulation tissue formation has also been demonstrated in zebrafish, where the wound is almost completely devoid of fibroblasts if FGF signaling is blocked [12]. Among other extracellular matrix molecules, fibroblasts secrete collagen type I, which is the most prominent constituent of muscle. IGF-1 is important for muscle regeneration partially through stimulating proliferation and differentiation of satellite cells [165, 475].

Materials and methods

Experimental set-up

The challenge/wounding trial was carried out at the infection facilities at BioMar A/S, Hirtshals, Denmark. The fish were kept in cylindrical 200L plastic tanks with a conical bottom for collection of waste. At the onset of the experiment each tank contained 180 fish with an initial weight of 53.6±11.8g and an initial length of 16.3±1.4cm (FL). All tanks were connected to the same filter and aeration system, and outflowing water underwent rigorous UV sterilization before re-entering the tanks. The fish were kept at a 12:12 light:dark cycle and at 15°C.

	No challenge (n=3)	Challenge 1 (n=2)	Challenge 2 (n=4)
Unwounded control fish	130	110	60
Trojans		20	20
Punch wounded	50	50	
1x Scrape			50
2x Scrape			50

Table 3.2.1. Number of differently treated fish in each tank for the experiment. 2-4 replicate tanks were used for each combination of treatments (n).

Wounding

Prior to wounding the fish were anaesthetized in 50mg/L MS-222. For each No challenge and Challenge 1 tank, 50 fish were wounded with a 6mm biopsy punch in an area anterior the dorsal fin and above the lateral line on the left side of the fish. The wound penetrated 3-4mm into the muscle. For the Challenge 2 tanks, 50 fish were scraped with a microscope glass slide on one side (1x Scrape), and 50 fish were scraped on both sides (2x Scrape). The affected area was approximately 2x3cm and located in the same position as the biopsy punch wounds, and disrupted the epidermis and removed most of the scales.

Challenge

Simultaneous to the wounding, 20 fish were anaesthetized similar to the wounded fish and injected intraperitoneally with 10^6 CFU of *Vibrio anguillarum* in phosphate-buffered saline.

Wound closure

Images were acquired on days 6, 12 and 29 after wounding with a multispectral imaging device called a VideometerLab (VideometerLab A/S, Hørsholm, Denmark). An advantage of the VideometerLab is its ability to acquire standardized images. The wound edge was manually outlined on the images, and wound size was measured in MatLab (MathWorks). The fish were anaesthetized as previously described prior to image acquisition, except for the fish that were sacrificed for gene expression studies. These were instead killed in an overdose of MS-222.

Gene expression

Muscle was sampled from biopsy punch wounded fish on days 3, 12 and 29, but the samples from day 3 were lost. 5 fish from each treatment (No challenge and Challenge 1) were sampled at each sampling day. Muscle samples were collected from the wound as well as a similar area on the opposite side of the fish (internal control) using an 8mm biopsy punch and removing any skin before immersing in RNAlater® in cryotubes. The samples were kept at 4°C or on ice for 24h, and then at -20°C until further processing.

The protocol for extraction of RNA, cDNA production and qPCR is described in **paper III**. All OD260:280 values were between 2.03 and 2.17.

The primer set for Collagen type I α1 chain is described in **paper III**, and the primers for IGF-1 and FGF-2 were designed with the Primer3 software and supplied by Sigma-Aldrich (Brøndby, Denmark). The primers were tested for specificity by melt curve analysis.

Gene	Primer	Sequence (5'-3')	GenBank Acc. No.	Amplicon length
RPS20	Forward	AGCCGCAACGTCAAGTCT	AY953432	104
(reference)	Reverse	GTCTTGGTGGGCATACGG		
IGF-1	Forward	AGCGGTCATTTCTTCCAGTG	M95183.1	78
	Reverse	GAGGGTGTGGGTACAGGAGA		
FGF-2	Forward	ATGGCCACAGGAGAAATCAC	AY878375.1	149
	Reverse	TCCACGCTTCCGTTAGAGTT		
Col1α1	Forward	TGAGGGAACTCCTGGTAACG	CK897549	74
	Reverse	ACTCACCACGTTCTCCCTTG		

Table 3.2.2. Sequences of the primers used for gene expression in the experiment.

Results

The Trojan fish in both challenge experiments had close to 70% mortality, with the most deaths occurring between 2 and 4 days after challenge (fig. 3.2.1). Deep wounding with a biopsy punch did not affect mortality, whether in the presence of *Vibrio anguillarum* or not (0% mortality). In Challenge 2 there was a surprisingly high mortality of the unwounded control fish (about 35%), and this was even slightly higher than for the scratch-wounded fish (25 and 28%).

Vibrio anguillarum did not significantly affect wound closure of the biopsy punch wounded fish (Fig. 3.2.2).

The expression of IGF-1 was downregulated in the wound relative to internal control at day 12, but had returned to normal at day 29 (Fig. 3.2.3) in challenge and control groups. The downregulation was significantly affected by the bacterial challenge and was greater in the control fish compared to the cohabitants. FGF-2 was upregulated in wounds on day 12, and on day 29 FGF-2 expression was still significantly higher in the wounds than in control muscle of cohabitants. Challenge increased FGF-2 upregulation in wounds. There was a limited effect on expression of Col1a1, but it was significantly upregulated in the wound at day 12 in the challenged group.

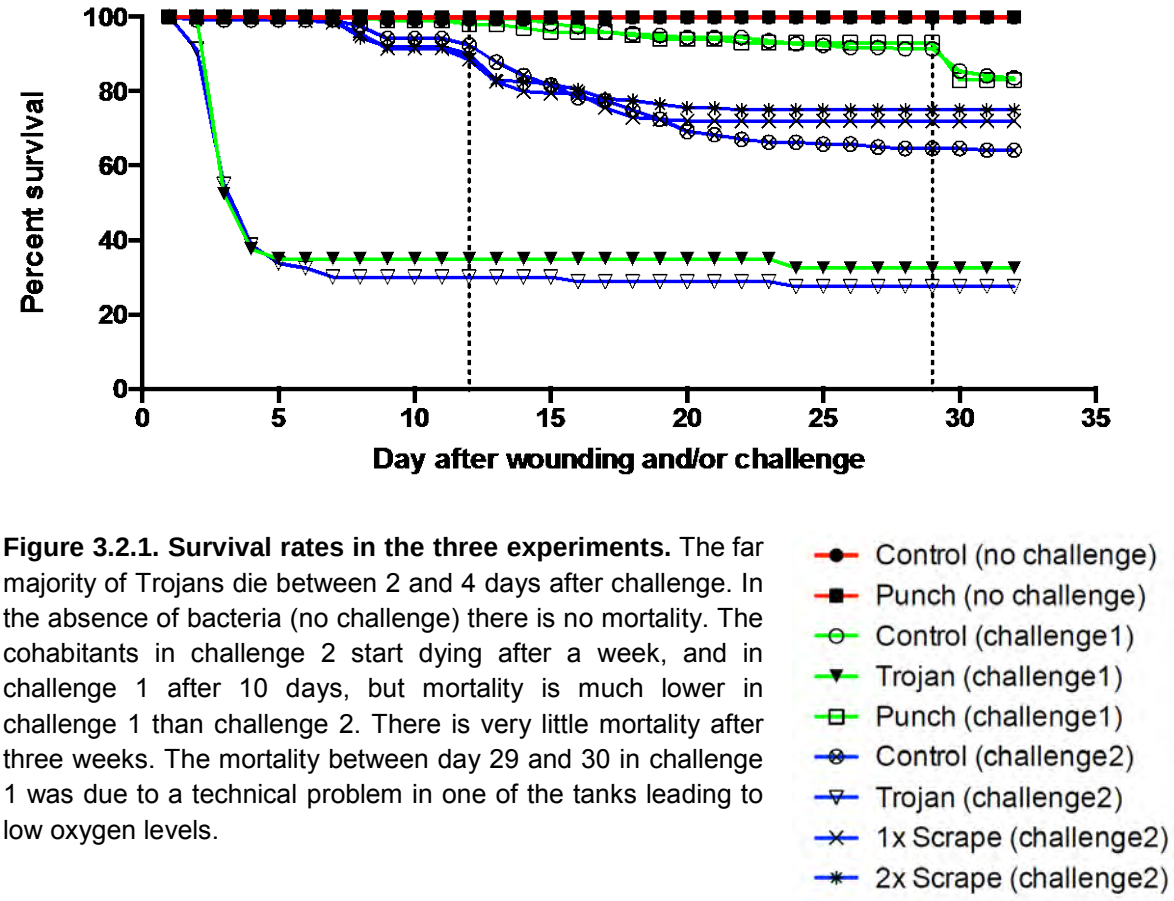


Figure 3.2.1. Survival rates in the three experiments. The far majority of Trojans die between 2 and 4 days after challenge. In the absence of bacteria (no challenge) there is no mortality. The cohabitants in challenge 2 start dying after a week, and in challenge 1 after 10 days, but mortality is much lower in challenge 1 than challenge 2. There is very little mortality after three weeks. The mortality between day 29 and 30 in challenge 1 was due to a technical problem in one of the tanks leading to low oxygen levels.

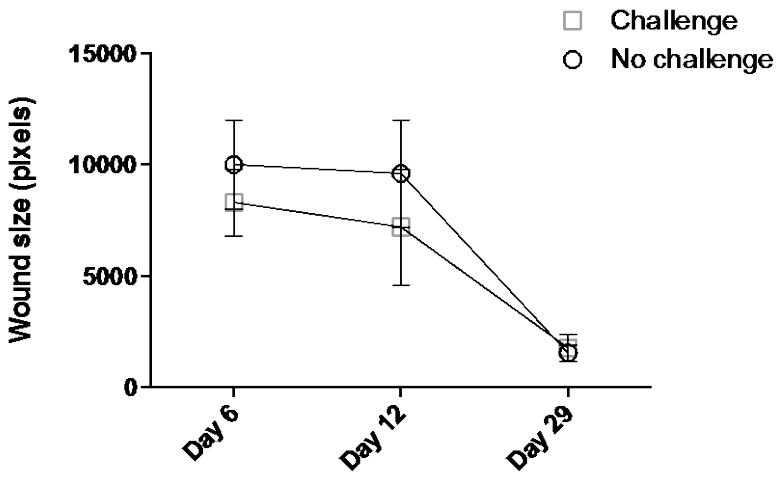


Figure 3.2.2. Wound size. The size of the open wounds were measured on days 6, 12 and 29 after wounding. There was no significant difference between the challenge and non-challenge groups.

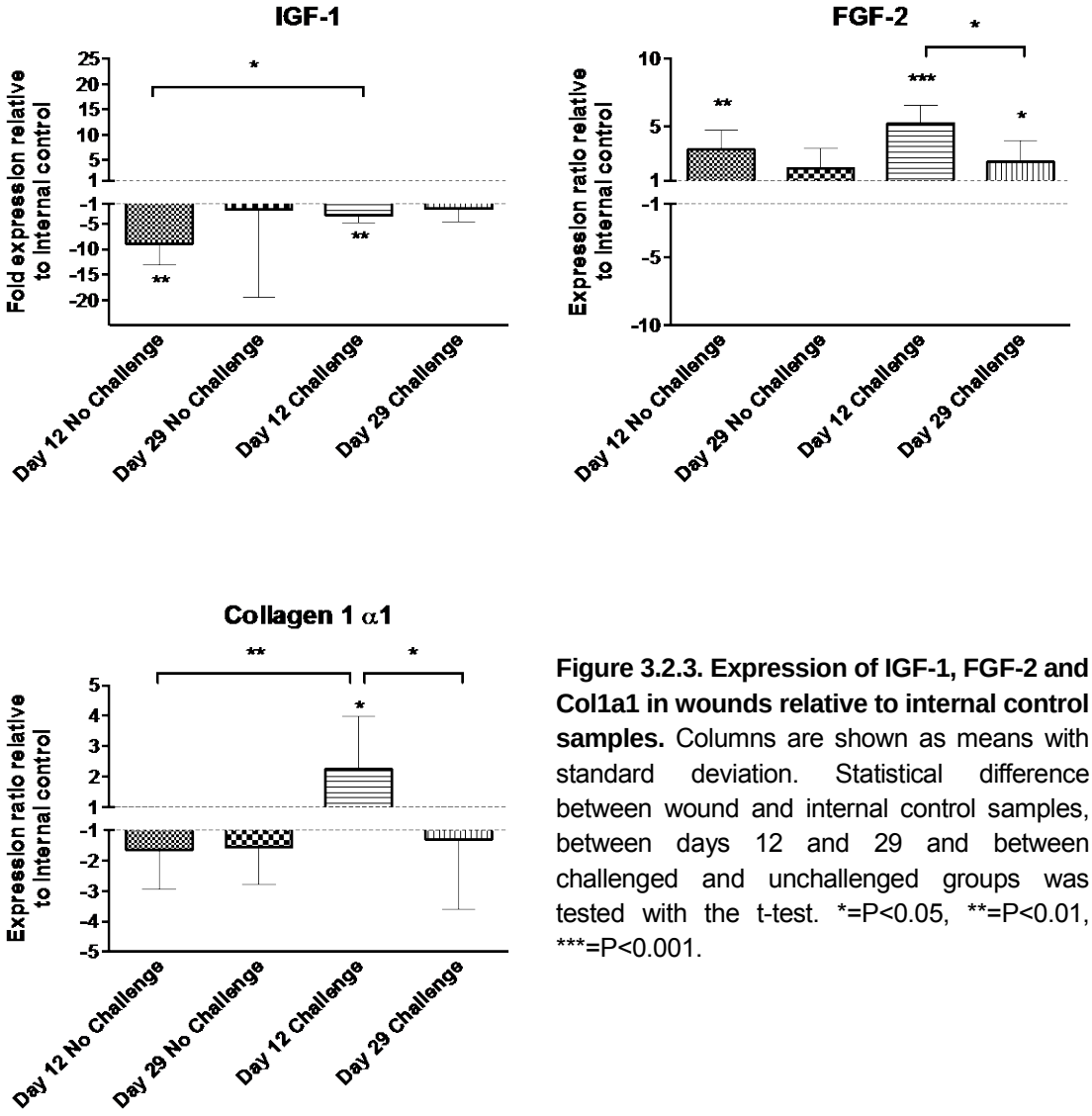


Figure 3.2.3. Expression of IGF-1, FGF-2 and Col1a1 in wounds relative to internal control samples. Columns are shown as means with standard deviation. Statistical difference between wound and internal control samples, between days 12 and 29 and between challenged and unchallenged groups was tested with the t-test. *=P<0.05, **=P<0.01, ***=P<0.001.

Discussion

The bacterial load is presumably highest around the time of death of the Trojans, and the infection pressure in the tanks is thus highest around day 3 or 4. Considering the re-epithelialization rates of fish wounds, the smaller (albeit deeper) punch-wounds were probably completely re-epithelialized at this time, whereas the larger scratch-wounds were not yet covered by epidermis. The scratch-wounded fish may thus have been lethally or sublethally infected and may have acted as secondary seeder fish, which may explain the high mortality in the challenge 2 experiment.

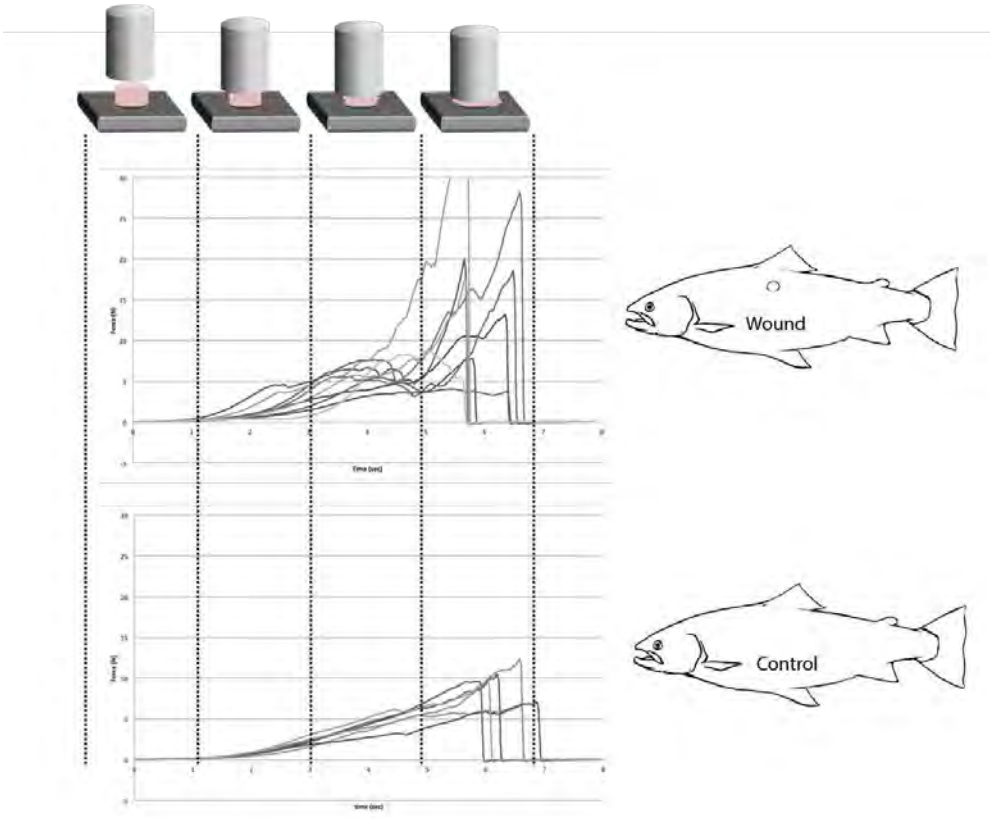
Ingerslev et al [11] demonstrated that rainbow trout infected with *Vibrio anguillarum* had more tough and fibrous fillets seven months after infection. We could thus expect that the challenge results in increased expression of collagen type I. However, we did not expect this upregulation to happen as early as 12 days post-wounding, as the initial wound matrix is dominated by non-collagen molecules.

The expression of IGF-1 and FGF-2 is higher in the wounds of fish challenged with *Vibrio anguillarum*. It thus seems that the bacterial challenge stimulates muscle repair and fibrosis. This is consistent with the observations by Ingerslev et al [11].

We were not able to demonstrate an effect of challenge on wound closure. However, the wound closure kinetics was subject to a high individual variability. It thus takes a large sample size to observe significant differences in wound closure.

The difficulties in controlling and standardizing the pathogen load in infection trials contributed to our decision of turning our focus to stimulation with non-infectious agents such as heat-killed bacteria and purified PAMPs.

3.3 Rainbow trout muscle texture is still affected by excisional wounding one year later



Graphical illustration of the compression of a cylinder of tissue (top) with a Texture Analyzer. The plots illustrate the compression force (Newton, Y-axis) as a function of time (seconds, X-axis) of wounded (top) and control (bottom) tissue. Notice the irregular curve pattern of wounds as a result of slipping of the tough tissue. These sudden changes in compression resistance are not observed for the softer control tissue.

- Excisional wounding had long-term effects on texture in rainbow trout. The wound site was fibrotic one year after excisional wounding, and more force was needed to compress post-wounded muscle tissue than control tissue from a non-wounded fish.

Introduction

Salmonids are important aquaculture production animals. In aquaculture, injuries and intramuscular bleedings can results from size sorting, biting and abrasion or from predators,

parasites and pathogens. Intramuscular bleedings result in inflammation and can lead to subsequent darkening (melanogenesis) of the fillet [476]. This leads to downgrading. Apart from the visually unappealing properties of fillets with a history of tissue damage, wound repair is associated with fibrosis in adult higher vertebrates. However, very little is known about injury-associated muscle fibrosis in fish. In zebrafish, initial fibrotic muscle tissue is apparently completely regenerated within a couple of weeks [12]. However, this observation was a purely visual evaluation. Apart from a recent experiment by Ingerslev and co-workers [11] there has been no focus on the effect of muscle damage on texture – an important sensory parameter for the consumer. Ingerslev et al [11] showed that that previous muscle injury (multiple needle puncture) in rainbow trout had no effect on textural properties 7 months post-wounding when the fillets were cold-smoked or heat-treated and evaluated by a sensory panel [11].
In this experiment we instead used a texture analyzer to measure texture of muscle samples taken locally in the wound area of fresh post-rigor fillets.

Materials and methods

Experimental set-up
The fish used in the present experiment were 31 fish in surplus from the experiment in **Paper III**. When the experiment in **Paper III** was terminated after 100 days, the remaining fish were PIT-tagged to identify treatment (± β-glucan and ± wound) and pooled in a 600L tank in the same facility under the same conditions, except the temperature was higher and more stable (14.0±1.7°C) for the remainder of the experiment. The fish were kept until one year (365 days) after the original experiment was initiated. The fish were then killed in 200mg/L MS-222, followed by a sharp blow to the head. The fish were photographed, measured (40.8±2.6cm) and weighed (1090±193g). Finally the ventral aorta was severed and the fish was left in ice-water for 5-10mins to bleed before being placed upright at 2°C. The sampling was carried out in December and the fish were committed to sexual maturation. It was thus clear from external morphological traits that out of the 31 fish, three were male. The males were sampled, but not included in the analysis in order to optimize homogeneity of the sampling groups. After 4 days when the fish were in post-rigor they were brought to the lab on ice. The wound area was still clearly visible, since scales had not regenerated in the affected areas (fig. 3.3.1) and a cylinder of tissue was removed from the wound area with an 8mm biopsy punch. Once removed from the fish the cylinder was trimmed to approximately 5mm. It was then placed with the skin side down (the skin was still attached to the muscle), and texture was measured immediately after to

avoid heating and drying out. Texture was measured using a Texture Analyzer TA-XT2 (Stable Micro Systems, Surrey, England) fitted with a flat-ended 15mm stainless steel cylinder probe set to move at 0.5 mm s^{-1}. Compression force was measured every 5ms until the tissue was compressed to 70% of its original height.

Data analysis

Since the original experiment (**Paper III**) showed no effect of β-glucan treatment the two groups were pooled, and thus only two groups remained: Wounded and control.

Due to the heterogeneous temporal changes in physical behaviour of the muscle under compression, it was decided that maximum force (N_{max}) was the most consistent measure. An unpaired two-tailed t-test was used to compare N_{max} of wounded and control groups.

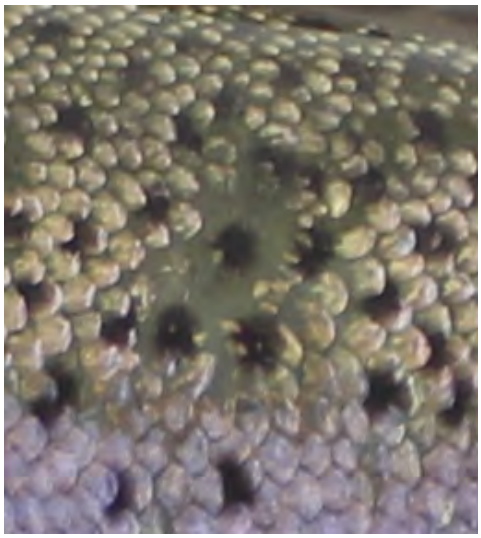


Figure 3.3.1. The scales had not regenerated one year after excisional wounding in any of the rainbow trout. However, pigmentation resembled that of unwounded skin. An example is shown here.

Results and discussion

The muscle tissue taken from wounded areas on average required almost twice as much force to compress 70% (fig. 3.3.2). This difference was statistically very sound (P=0.0144). The large standard deviation in the wounded group can be explained by the occasional slipping of the sample under the flat cylinder during compression (see graphical illustration at the beginning of section 3.3). This was more common for the firmer wounded tissue, and led to an underestimation of the required compression force. Thus, the actual difference may in fact have been larger than observed.

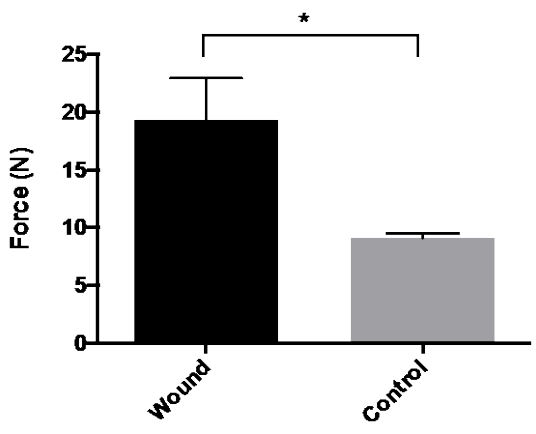


Figure 3.3.2. shows the average maximal force (measured in Newton; N) required for compression of wound tissue one year after wounding compared to unwounded fish kept under the same conditions. There is a significant difference between the two groups (P=0.0144).

4. Discussion and conclusions

We are just beginning to scratch the surface of muscle wound healing in fish. Our experiments show that deep excisional cutaneous wounding has long-term effects on muscle texture and on the visual appearance of the skin.

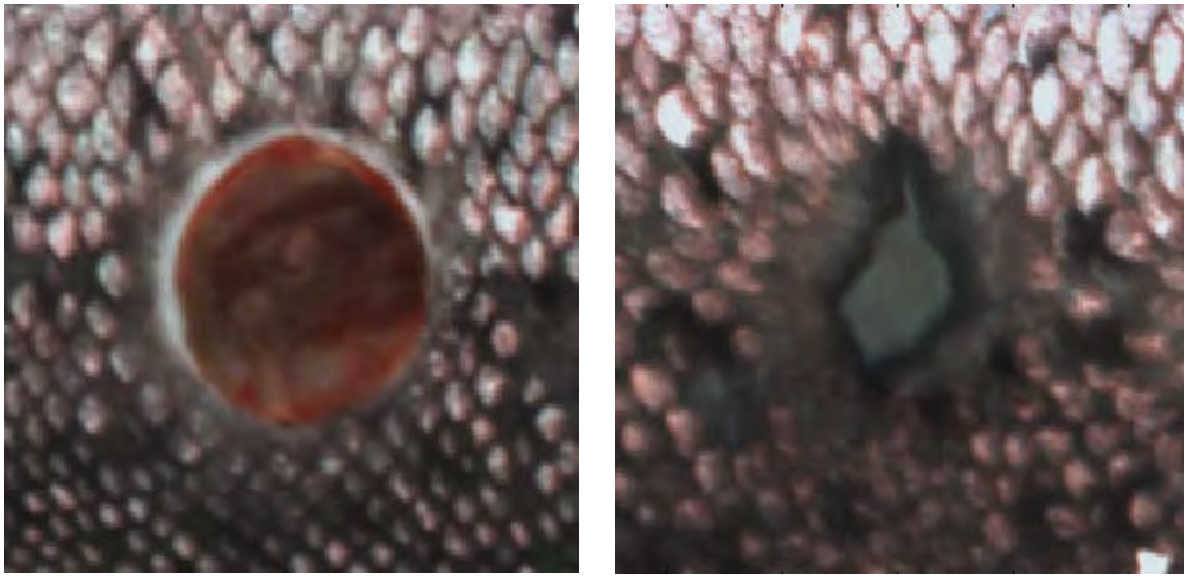


Figure 8. As the wound healing progresses the wound contracts. This results in distortion in the otherwise regular scale pattern. The image on the left shows a full-thickness excisional cutaneous wound from a rainbow trout 14 days post-wounding. The image on the right is acquired 100 days post-wounding. The images are from two different specimens.

The influence of previous muscle damage on sensory properties of the fillet has received relatively little attention in the aquaculture industry as well as the scientific community. Several handling practices introduce tissue damage. Fish are particularly prone to damage when handled out of water and suffer contusions during sorting procedures. Fish producers are well aware that such procedures can result in lethargy and reduced apetite in the following days, but less aware of the long-term influence on the quality of the final consumer product. Optimal production systems and gentler handling will thus not only increase fish welfare, but also fish growth, profit and product quality.

The overriding observations across all the conducted experiments are: 1) There is a large individual difference in wound closure dynamics in both of the two investigated species; and 2) wound closure dynamics are apparently to a large extent difficult to affect. These two observations leads to the conclusion that large experimental groups are generally needed to

show a statistically significant effect of experimental wound closure manipulations. Apart from the presented experiments, we also investigated the effect on wound closure of different feed types or of exposure to heat-killed pathogenic bacteria. None of these affected wound closure (results not shown).

The bullet points on the following pages provide an overview of important findings of the PhD study. These are presented in no particular order of importance.

1. Bathing in β-glucan-containing products stimulated wound healing in common carp (**Paper I**) as measured by visual wound closure dynamics of open full-thickness excisional wounds to the trunk. A similar experiment on rainbow trout (**Paper III**) resulted in no effect of β-glucan stimulation. Whether temperature (8.5±1.7°C for the rainbow trout and 21±1°C for the carp) or species was mainly responsible for this different outcome could not be established. However, the rainbow trout from **Paper III** had considerably longer wound closure kinetics than trout kept at 15±1°C (**section 3.2 and 3.3**) (wound closure kinetics from these experiments have been combined in fig. 9).

2. Carp wounds apparently closed with a much higher degree of contraction than rainbow trout wounds did. This may be related to the structure of the skin. The stratum compactum is less well developed in cyprinids than in the salmonids [477], and it is known from mammals that wound closure in tight-skinned species (*e.g.* humans) is mainly due to re-epithelialization, whereas in species with a loose skin structure (*e.g.* rodents) it is mostly by contraction [16].

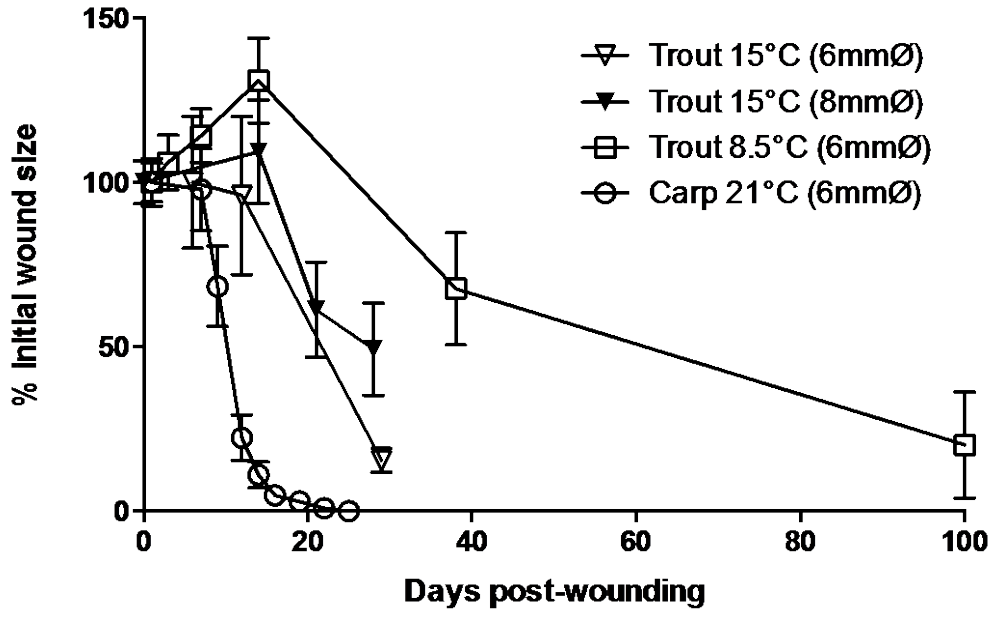


Figure 9. Wound closure kinetics of rainbow trout and carp at different temperatures. Difference between wound closure of 6mm and 8mm diameter wounds at 15°C is also shown.

3. At 15°C cohabitation challenge with the fish-pathogenic bacterium *Vibrio anguillarum* did not significantly affect mortality or wound closure dynamics, but did result in a higher expression of collagen type I and IGF-1 in the wound. This indicates a profibrotic effect of the challenge.

4. Larval and juvenile carp respond with very limited inflammation and regulation of immune-relevant genes when wounded by incisional needle piercing of skin and muscle. However, the immunoglobulin isotype IgZ1 is upregulated by the wounding at the larval stage. The wounds heal with visually perfect regeneration within 3 days in larval carp. After metamorphosis the wound site is still visible even after 7 days.

5. Healing of full-thickness excisional wounds is slow in rainbow trout kept at 8.5±1.7°C (**Paper III**). Gene expression data indicate that the inflammation phase (IL-1β expression) peaks around 14 days after injury, and is almost resolved by day 38. The expression of genes relevant for ECM production is initially downregulated, but is upregulated from one week after injury. The only exception to this is collagen type I α1-chain, the upregulation of which is delayed until day 38.

6. **Paper III** is the first report of gene expression of LOX, P4H and CD163 in fish, and until recently also of TGF-β3 [344]. LOX and P4H expression may be useful markers of muscle texture properties in future studies. CD163 expression correlated with expected presence of M2 polarized macrophages, and further investigations may validate CD163 is a valuable marker of fish M2 polarized macrophages.

7. Rainbow trout muscle does not regenerate following excisional wounding. Instead the healing wound is fibrotic, and there is a long-term effect on muscle texture. When texture is measured by compression with a texture analyzer one year after wounding, post-wounded muscle is significantly harder to compress than control muscle. This was not investigated in carp, but in this species the wound area was still raised, darker and harder to the touch relative to un-wounded areas one year after wounding (personal observations).

8. The number of melanin-based skin spots negatively correlates with wound closure in rainbow trout. The number of skin spots has been shown by others [471] to correlate

with the stress response, but it remains to be demonstrated if the effect of number of skin spots on wound healing observed here is linked to the stress response.

9. Scales at the site of excisional wounding did not show signs of regeneration in rainbow trout up to one year after the procedure (fig. 3.3.1), whereas carp scales regenerated within a few weeks, although not perfectly (fig. 11). However, these two situations cannot be directly compared since only the scale was removed in the carp, not the underlying skin and muscle. Skin pigmentation returns to almost normal in both species, but the affected area remains slightly darker than the surrounding skin.

10. It was observed that carp commonly bled profusely following wounding (fig. 10), whereas bleeding was more limited or in some cases appeared completely absent in rainbow trout, which thus more resembled the limited hemostasis also observed when inflicting wounds in the axolotl [37]. The implications of this were not investigated further. However, a blood clot apparently has no effect on wound healing in zebrafish [12].

Figure 10. Extensive bleeding from a biopsy punch wound on the flank of a carp immediately after it was inflicted.

Figure 11. Scale regeneration in carp. In a mirror carp with unusually many scales, several scales were removed prior to biopsy punch wounding. These regenerated within the same time frame as wound closure. However, the scales were distorted at the site of insult, whereas a neighboring scale not affected by the wound regenerated perfectly. D is day after wounding.

5. Perspectives for future research

Wound contraction
It was difficult to identify and outline the wound edge accurately at later stages in wound healing due to hyperpigmentation of the wound area. It could thus be valuable to instead develop a method to estimate wound contraction by analyzing the distortion in the otherwise very regular scale pattern in rainbow trout (see fig. 8).

Another interesting observation regarding wound contraction was made in carp in which anterior wounds healed with more contraction compared to more posterior wounds. We speculate that this is due to the increase in body movement towards the tail during swimming, and thus in more strain put on the wound matrix. Different wound locations could thus provide interesting clues to the molecular basis for differences in wound contraction in carp.

Melanomacrophage centers?
Another interesting observation is the temporal presence of dark spots in the periphery of the wounded area in carp. These spots typically start forming two to three weeks after wounding, and persist for three to four weeks before disappearing again. We speculate that these dark spots could be melanomacrophage centers (MMCs), which have been proposed to be primitive analogs of mammalian germinal centers [450]. At the present MMCs are most often often studied histologically in the kidney, which requires sacrificing the fish. It also makes it difficult to study the kinetics of their formation. Thus, if these spots are indeed MMCs, they could provide valuable information on B-cell development and antibody responses, since they can be easily visually observed and studied *in vivo* at different time-points in their lifetime from formation to dispersion. The spots can be harvested with minimal distress for the fish, and be used for gene expression studies, immunohistochemistry or other purposes. This information could potentially be used to improve vaccine efficacy in aquaculture.

Effect of β-glucan
β-glucans promoted wound contraction in carp (**Paper I**), but had no effect on rainbow trout (**Paper III**) wound healing. The reasons for the differing effects of β-glucan in the rainbow trout and carp study could not be determined. It would be interesting to repeat the experiment for the trout at a higher temperature and carp at a lower temperature to establish whether the response to PAMP stimulation is temperature and/or species dependent.

Thrombocyte function

CD41 expression levels in the wound peaked on the first day after wounding in rainbow trout, but expression levels were consistently elevated for at least two weeks after wounding (**Paper III**). This could indicate a persistence of thrombocytes in the wound. Since thrombocytes are possibly antigen presenting and phagocytosing in rainbow trout [184, 190, 191], it would be interesting to take a closer look at the involvement of thrombocytes in later stages in wound healing.

M2 marker?

CD163 upregulation in the wound of rainbow trout coincided with resolution of inflammation at a time when the M2 macrophage phenotype would theoretically predominate in the wound (**Paper III**). CD163 is a marker of mammalian M2c macrophages, but it is not known if this is also the case in fish. There is a general lack of fish M2 macrophage markers. It would thus be interesting to investigate closer the expression of this gene in isolated and properly stimulated macrophages, as well as in different cells and tissues to hopefully establish CD163 as a marker of M2 macrophages also in fish.

Skin spots

We found that the number of melanin-based spots in the skin of rainbow trout correlated positively with wound size (**section 3.1**). In other words, fish with many spots were slower healers. Kittilsen et al. [471] demonstrated that the salmonid stress response was lower in fish with many skin spots compared to fish with few skin spots. We were not able connect the final dots and establish if stress does indeed speed up wound healing, since we did not measure cortisol levels or other stress parameters. However, when we exposed rainbow trout to bacterial challenge we subjected them to stress (**section 3.2**). We did not see an effect on wound closure, but wound closure is subject to large individual variation, and we had only few fish. However, the gene expression was consistent with a faster healing, perhaps with fibrosis as a trade-off. Stress may thus promote wound healing at the expense of fibrosis in fish. This would go against the observations from mammals, which show that stress impairs wound healing. Furthermore, aquaculture itself can be stressful for the fish, and how stress affects wound healing and fibrosis would be of interest for the aquaculture industry, since it influences not only fish welfare, but also the quality of the final product. Thus this subject would be interesting to pursue.

Although the correlation was significant in our experiment, it was quite weak. Since the regulation of tyrosinase expression is very much influenced by temperature in the Atlantic salmon head kidney-derived cell line HKL-1 [474], it would be worth to repeat the experiment at a higher temperature and sample blood for cortisol measurement to correlate wound healing with the stress response. To exacerbate the stress response it would also be valuable to include another stressor apart from the elevated temperature and the wounding procedure itself. This experiment could be coupled to the proposed repetition of the PAMP-bathing experiment to reduce the use of experimental fish.

Melanophores

It appears that the melanin producing cells melanophores and melanomacrophages are associated with inflammation in skin and muscle respectively [8, 45, 61, 476]. The immune-related properties of macrophages are well described, but not much is known about the involvement of the melanophore. It may be that melanophores contribute with no more than the anti-oxidant effect of melanin [474], but it would be interesting to investigate if melanophores had immune-related properties, perhaps by contributing with cytokines and growth factors.

Wound type

We used excisional wounding as the overriding method of wounding in our experiments. This is a good model for wounds created by for example leeches, fish lice (*Argulus* spp. and *Lepeotheirus salmonis*) and lampreys. It would be interesting to also investigate the effect of other types of wounds on textural, visual and molecular parameters. Fish out of water are susceptible to contusions – *e.g.* during vaccination and size sorting procedures. Since all production fish are usually handled at some time points during their lifetime, contusions are likely a very relevant form of wounding in aquaculture, and one that is relevant to investigate. Contusions are truly sterile since the mucosal surface is not breached, as opposed to open wounds that are never entirely sterile. They may thus be more suitable to investigate the effect of PAMPs versus DAMPs.

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List of abbreviations

AA:	Arachidonic acid
CCR:	CC Chemokine receptor
CD:	Cluster of differentiation
CTGF:	Connective tissue growth factor
DAMP:	Damage-associated molecular pattern
DHA:	Docosahexaenoic acid
ECM:	Extracellular matrix
EPA:	Eicosapentaenoic acid
EST:	Expressed sequence tag
FAP:	Fibrocyte/adipocyte precursor
FGF:	Fibroblast growth factor
FN:	Fibronectin
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
HA:	Hyaluronic acid
HGF:	Hepatocyte growth factor
HMGB1:	High mobility group B1
HPI-axis:	Hypothalamus-pituitary-interrenal-axis
ICAM:	Intercellular adhesion molecule
IFN:	Interferon
Ig:	Immunoglobulin
IGF-1:	Insulin-like growth factor 1
IL:	Interleukin
ILC:	Innate lymphoid cell
iNOS:	inducible nitric oxide synthase
KGF:	Keratinocyte growth factor
LOX:	Lysyl oxidase
LPS:	Lipopolysaccharide
LTi cell:	Lymphoid tissue inducer cell

MAb:	Monoclonal antibody
MC/EGC:	Mast cell/eosinophilic granular cell
MHC:	Major histocompatibility complex
MMP:	Matrix metalloproteinase
MRF:	Muscle regulatory transcription factor
MS-222:	Tricaine methanesulfonate
NCC:	Natural cytotoxic cell
NET:	Neutrophil extracellular trap
NH cell:	Natural helper cell
NLRP3:	NOD-like receptor family, pyrin domain containing 3
NO:	Nitric oxide
NSAID:	Non-steroidal anti-inflammatory drug
P4H:	Prolyl 4-hydroxylase
PAMP:	Pathogen-associated molecular pattern
PBL:	Peripheral blood leukocytes
PDGF:	Platelet-derived growth factor
PGE2:	Prostaglandin E2
PRR:	Pattern-recognition receptor
RORγt:	Retinoic acid receptor-related orphan receptor gamma t
ROS:	Reactive oxygen species
TAP:	Transporter associated with antigen processing
TGF-β:	Transforming growth factor-β
Th cell:	Helper T cell
TLR:	Toll-like receptor
TN-C:	Tenascin-C
TNF:	Tumor necrosis factor
VEGF:	Vascular endothelial growth factor
α-SMA:	alpha-smooth muscle actin

Accompanying papers

Paper I

β-glucan enriched bath directly stimulates the wound healing process in common carp (*Cyprinus carpio* L.)

Przybylska, D. A., Schmidt, J. G., Vera-Jimenez, N. I., Steinhagen, D., & Nielsen, M. E.

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Short communication

β-glucan enriched bath directly stimulates the wound healing process in common carp (*Cyprinus carpio* L.)

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ABSTRACT

Wound healing is a complex and well-organized process in which physiological factors and immune mechanisms are involved. A number of different immune modulators have been found to enhance the non-specific defence system in vertebrates, among which β-glucans are the most powerful and extensively investigated.

The aim of the present study was to investigate the biological impact of two different commercially available β glucan containing products on the wound healing process in carp. Throughout a two week experiment fish were kept either untreated (control), or in water supplemented with the two different types of β-glucans. The wound healing process was monitored using a multispectral visualisation system. The correlation between wound closure and immune response was investigated by measuring the gene expression patterns of IL-1β, IL-6 family member M17, IL-8 and Muc5b, and measurement of production of radical oxygen species. PAMPs/DAMPs stimulation caused by the wounding and or β-glucans resulted in an inflammatory response by activating IL-1β, IL-6 family member M17 and IL-8 and differences in the expression pattern were seen depending on stimuli. IL-1β, IL-6 family member M17 and IL-8 were activated in all wounds regardless of treatment. Expression of all three interleukins was highly up regulated in control wounded muscle already at day 1 post-wounding and decreased at subsequent time-points. The reverse was the case with control wounded skin, where expression increased from day 1 through day 14. The results for the β-glucan treated wounds were more complex. The images showed significantly faster wound contraction in both treated groups compared to the control. The obtained results clearly demonstrated that a β glucan enriched bath promotes the closure of wounds in common carp and induce a local change in cytokine expression.

1. Introduction

Skin, together with the mucus, forms the first line of defence against pathogens and is an essential protective barrier in aquatic organisms. Mucus can entrap foreign particles and microorganisms, before they can interact with the epithelial surface and cause damage [1]. The main mucus components are water and large, highly glycosylated glycoproteins called mucins. Mucins and mucus have rheological properties (viscosity and elasticity), which are important for their physiological function [2]. Based on biochemical characterisation, 19 mucin genes have been identified in humans and gene structure as well as their role in the infection process has been described [3–5]. To date, only two mucin genes, Muc2c and Muc5b, have been cloned and sequenced in fish [6].

Wounds in fish can be caused by pathogens such as ectoparasites or Gram-negative bacteria, as well as by physical trauma [7–9]. In vertebrates reduced skin integrity or mucus production facilitates entry of pathogens into the underlying tissue and vascular system, therefore rapid reaction at the wound site is essential [10]. Wound healing is a complex and well-organized process which can be roughly divided into three overlapping phases: inflammation, tissue formation and remodeling, in which blood cells, soluble mediators, resident cells (fibroblasts, endothelial cells, goblet cells) and extracellular matrix components are involved [10–12].

The immunomodulatory effect of β-glucans has been shown in studies on several taxonomic groups including fish [13–18]. These naturally occurring polysaccharides differ in length, molecular weight, extent of branching and bioactivity, and in vertebrates trigger different biological effects [19–24]. β-glucans have been

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shown to accelerate the wound healing process in mammals, however such effect remains to be proven in fish [25,26].

β-glucans are pathogen-associated molecular pattern molecules (PAMPs) and are recognized by pattern recognition receptors (PRRs) such as toll-like receptors (TLR) or NOD-like receptors (NLRs), and activate transcription of pro-inflammatory genes [27]. Furthermore, PRR signaling can also be activated by "danger signals" – damage associated molecular patterns (DAMPs), which are endogenous molecules such as nucleic acids, high-mobility group box 1-protein or heat shock proteins released from cells during tissue damage, or hyaluronan products degraded during said process [28,29]. Hence, introducing PAMPs and DAMPs simultaneously may have a combined impact [30].

Mammalian models have shown that in response to PAMPs and DAMPs, numerous cytokines are secreted and subsequently, will guide and selectively activate leukocyte subsets [12]. Some cytokines have shown to be highly important during wound healing. Mammalian interleukin 1β (IL-1β) is strongly up-regulated during the inflammatory phase of healing [31,32]. The main IL-1β sources are polymorphonuclear leukocytes (constitute nearly 50% of all cells at the wound site in first few days post-wounding) and macrophages, as well as some resident cells [12,31]. In addition, human recombinant IL-1β is reported to affect mucus release in mice [33]. Interleukin 8 (IL-8) is a known regulator of neutrophil trafficking and augments angiogenesis in mammals [12,32,34–36]. Carp possess two CXCL8 lineages, which appear to be functional homologs to mammalian IL-8 [37]. Expression of IL-8 in mammals is correlated with interleukin 6 (IL-6) [38]. IL-6 is produced and secreted by macrophages, neutrophils, as well as resident cells [12,31]. Experiments on diabetic or IL-6 knockout mice have shown a reduction in neutrophil and macrophage number, as well as a decrease in collagen production and deposition [39,40]. Orthologues of the mammalian IL-6 have been identified in fish [41–44]. Moreover, PAMPs and DAMPs induce production and secretion of hydrolases, complement components, and reactive oxygen and nitrogen species (ROS and RNS) by macrophages in vertebrates [45–48].

In the present study, we have examined the direct biological effect of β-glucan enriched products on the wound healing process in common carp in a controlled environment. This effect has been verified by monitoring visual wound contraction dynamics and gene expression patterns of three pro-inflammatory cytokines: IL-1β, IL-6 and IL-8, as well as measurement of ROS production. Additionally, as Muc5b is the major gel-forming mucin in the protective mucous matrix of mammals, its expression was investigated [49].

2. Materials and methods

2.1. Animals

One-year-old common carp (*Cyprinus carpio* L., R3xR8, WUR, The Netherlands) were kept in 30 L aquariums filled with tap water adjusted to 21 °C and fed a commercial carp feed (Trouvit, Nutreco) at 2% of their estimated body weight per day. Fish were exposed to a 12/12 light/dark cycle. The fish were divided into six groups (Table 1).

Table 1
Experimental groups.

		Water condition		
		Control	MacroGard	6.3 kDa fiber
Wounding	No wound	12 (3 tanks of 4 fish)	12 (3 tanks of 4 fish)	12 (3 tanks of 4 fish)
	5 mm Biopsy	12 (3 tanks of 4 fish)	12 (3 tanks of 4 fish)	12 (3 tanks of 4 fish)

2.2. Preparation of β-glucans

For stock solution preparation, 0.5 g of MacroGard (yeast, >60% pure, Biorigin, Brasil) or 6.3 kDa fiber (oat, 50% pure, Scan Oat, Sweden) was dissolved in 500 ml MilliQ water. In order to dissolve the soluble β-glucan fractions, solutions of both products were stirred for 1 h at 90 °C and autoclaved (121 °C, 15 min, 1 atm). In the experimental setup, water was changed daily and the β-glucan product concentrations were adjusted to 0.1 μg/ml.

2.3. Wounding and sampling procedure

Carp were anaesthetised by immersion in 0.01% benzocaine (VetPharm, Belgium). 5 mm Ø biopsy punches (Miltex, Inc, USA, Fig. 1.) were used to mechanically damage areas of the skin and musculature (left side, above the lateral line, three wounds per fish). At day one, three and fourteen post-wounding four individuals from each group were anaesthetised and, using 8 mm biopsy punches, the following tissue samples from the edges of the wounds were collected: 1. Muscle tissue from the wounded area; 2. Muscle tissue from a non-wounded area (right side); 3. Skin tissue from the wound area; 4. Skin tissue from non-wounded area (right side); 5. Muscle and skin tissue from all control fish (left side, Fig. 1). Samples were collected and immediately frozen in liquid nitrogen for further analysis. This experiment was conducted according to Danish legislation and by scientists accredited by the Federation of Laboratory Animal Science Associations (FELASA).

2.4. RNA isolation and cDNA synthesis for qPCR

The previously frozen in the liquid nitrogen samples (50 mg) were mixed with 500 μl of buffer (2-Mercaptoethanol in lysis buffer) and sonicated (30 s, pulse 2, amplitude 70%, using a soVCX-130 sonicator CiAB, Sweden) as it is considered as more straightforward method than homogenization [50]. Following the manufacturer's protocol, RNA was isolated using GenElute Mammalian™ Total RNA Miniprep Kit (Sigma–Aldrich, Denmark) and subsequently treated with DNase-I (Sigma–Aldrich, Denmark) to remove any genomic DNA. RNA purity and quantity was determined by OD260/280 measurements on a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Germany). 400 ng of total RNA was primed with random hexamers and reverse transcribed using TaqMan® Reverse Transcription reagents (Applied Biosystems, Denmark) in a final volume of 20 μl. The synthesized cDNA was diluted 1:10 in MilliQ water and stored at −20 °C until further analysis.

2.5. Real-time quantitative PCR

Based on sequences deposited in the GenBank (refer to Table 2) and using the program Primer3, PCR primer sets specific for 40S, IL-1β, IL-6 and IL-8, were designed [51]. The mucin 5b (Muc5b) primer set was provided by Fish Disease Research Unit at the University of Veterinary Medicine, Hanover, Germany. The subunit S11 of the ribosomal gene 40S was selected as a reference gene based on previous work [7]. The assays for examined genes were run using SYBR® Green JumpStart™ *Taq* ReadyMix™ (Sigma–Aldrich, Denmark). Quantitive RT-PCR was performed using a Stratagene MX3000P™ real-time PCR system (Integrated Sciences, Australia). Master mixes for each PCR run were prepared as follows: 12.5 μl Brilliant SYBR® Green JumpStart™ *Taq* ReadyMix™, 1 μl of each primer and 5.5 μl ultra pure water. Finally 5 μl of diluted cDNA was added to 20 μl of master mix. The cycling conditions for the reaction were: incubation step of 10 min at 95 °C, followed by 45 cycles of 95 °C for 30 s and 60 °C for 1 min. At the end of each reaction, DNA melting curve analysis was performed in order to

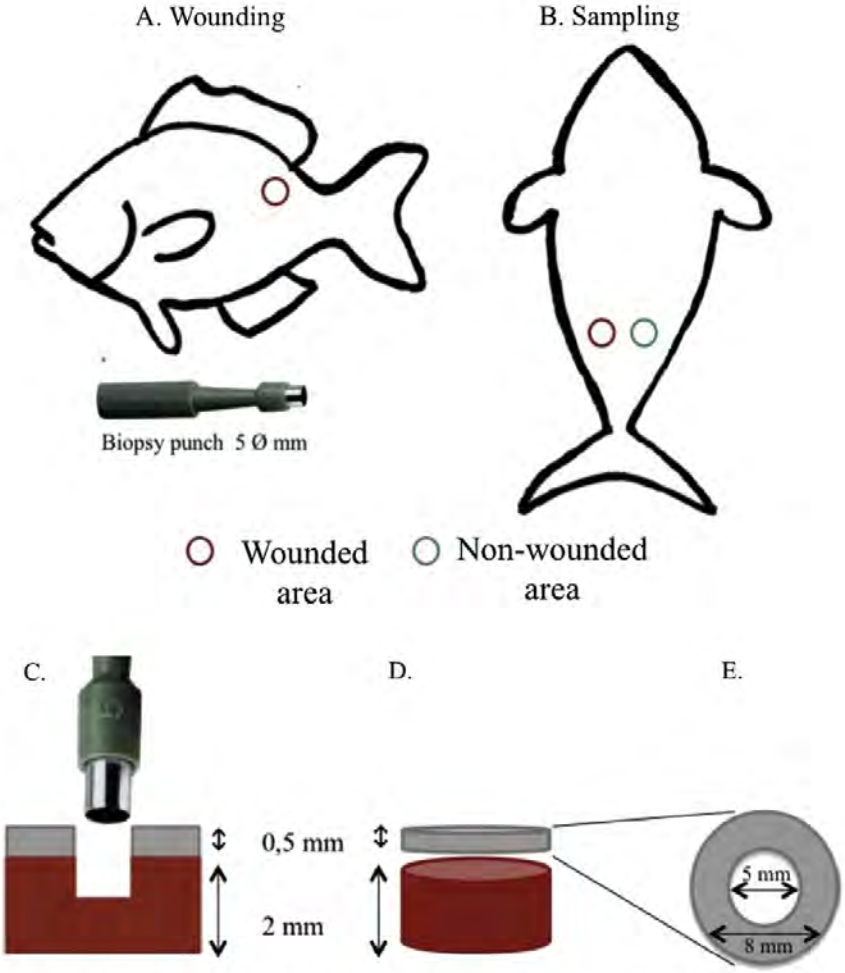


Fig. 1. Illustration of wounding (A) and sampling (B) procedures and biopsy punches. Fish were wounded on the left side above the lateral line (red circle). During sampling, skin and muscle tissue were taken from the wounded site (red circle), as well as from non-wounded site (internal control, green circle). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

confirm the specificity of the PCR products. All samples were run in triplicate, and for each primer set, control reactions without cDNA were included in order to detect any non-specific amplification. The expression results were analyzed using the $-2^{-\Delta\Delta Ct}$ method [52]. Data are shown as fold expression relative to non-wounded internal control site, and the analysis was carried out using Mx ProTM qPCR software (Integrated Sciences, Australia). The threshold values (Ct) were determined manually for each run.

Table 2
Sequences of primers used for real-time PCR.

Gene	Primer	Sequence (5′–3′)	GeneBank acc. No.
40S	Forward	GTTGAAGGAAGTGGCAAGGA	AB012087
	Reverse	AGAATACGGCCTCTGATGGA	
IL-1β	Forward	AAGGAGGCCAGTGGCTCTGT	AJ245635
	Reverse	CCTGAAGAAGAGGAGGCTGTCA	
IL-6 family member (M17)	Forward	CCGCACATGAAGACAGTGAT	AY102632
	Reverse	GGGTATATTTGGCTGCAGGA	
IL-8	Forward	TGGAGCTCTTCCCTCCAAG	EU011243
	Reverse	AGGGTGCAGTAGGGTCCAG	
Muc5b	Forward	CAGCCCTCTTCCTCTTTCATC	JF343438
	Reverse	CCACTCATCTTTCCTTTCTCTTC	

2.6. Isolation of head-kidney (HK) cells and measurement of respiratory burst

Anaesthetised fish were bled from the caudal vein. Both head-kidneys (HK) were excised and placed on a 100 μm nylon cell strainer (BD Falcon, New Jersey, USA). A cell suspension was obtained by pressing the head-kidneys with a plunger through the cell strainer, and rinsing them with phenol red-free Hank's balanced salt solution with 0.1% gelatine (g-HBSS). The HK cells were counted using a Bürker chamber. Cell viability was assessed by Trypan exclusion and cell concentration was adjusted to 3×10^6 HK cells/ml in g-HBSS. Subsequently, the respiratory burst activity was measured using luminol-amplified chemiluminescence, modified from the protocol described by Vernho et al. (2005) [53]. To elicit the respiratory burst response,

Aeromonas hydrophila were incubated overnight. The bacteria was washed twice with HBSS, OD_{600} was set to 0.5, and 50 µl was added to each well in a white 96-well plate (Sigma–Aldrich P8616, Denmark). Next, a mixture of 40 µl of 10 mM luminol in 0.2 M borate buffer (pH 9.0) and 100 µl of HK cells were added. The total well volume was adjusted with g-HBSS to 300 µl. The chemiluminescence emission from the HK cells was measured with a luminometer (Synergy2, Biotek) every 3 min for 210 min at 26 °C. Results are expressed as the integral of the relative light units (RLU) recorded by the luminometer (Max RLU).

2.7. Visual analysis of wound closure

Prior to sampling of the head kidney, skin and muscle, images of the wounds were acquired using a VideometerLab (Videometer A/S, Hørsholm, Denmark). The fish were placed in a special container in order to exclude the ambient light as the VideometerLab provides diffuse illumination from light-emitting diodes to capture 20 images with unique spectra between ultra violet and near infra red of the electromagnetic spectrum. The multispectral imaging facilitated the visual detection of the wound edge. The distance from the camera to the right (intact) side of the fish was kept constant. Differences in width of individual carp could affect the image capture area of the wound. However, given the long distance from the camera to the subject (~40 cm) and narrow size range of the fish, this had no measurable effect on wound size estimations. The wound edges on day 14 post-wounding were outlined and the resulting open wound area determined using a script in MATLAB (The MathWorks Inc., Natick, MA, USA) (Fig. 2).

2.8. Statistics

A non-parametric Mann–Whitney test was used to compare results from gene expression and respiratory burst. Differences between groups were regarded as significant at $p < 0.05$. Results from visual wound closure were tested with a two-way ANOVA using the Prism software, version 4.03 (Macintosh, GraphPad Inc., La Jolla, CA, USA).

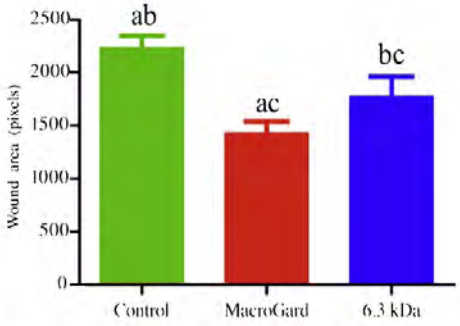


Fig. 3. Statistical representation of the wound sizes fourteen days post-wounding from wounded-control, wounded-MacroGard and wounded-6.3 kDa groups. Bars show mean values (pixels) + SD of $n = 4$ ($n = 3$ 14 days in wounded-MacroGard group). "a" and "b" depict statistical significance ($p < 0.01$) between control and MacroGard groups, and control and 6.3 kDa groups subsequently. "c" depicts statistical significance ($p < 0.05$) between MacroGard and 6.3 kDa groups.

3. Results

3.1. The visual healing of the wounds

At day 14 post-wounding, the wound size was significantly ($p < 0.01$) reduced in both β-glucan treated groups in comparison to control fish, but significantly more in the MacroGard-treated group. Fig. 3 shows the differences in open wound area 14 days post-wounding from the experimental groups.

3.2. Gene expression during wound healing process

No significant changes were observed in non-wounded groups.

3.2.1. Interleukin 1β (IL-1β)

In skin collected from the W-C group, IL-1β showed a tendency to increase over time (Fig. 4A). Muscle samples taken one day

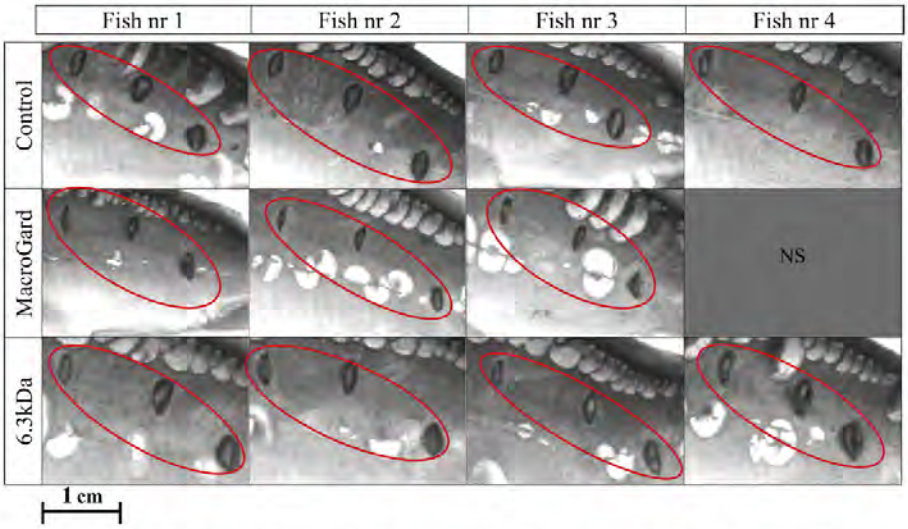


Fig. 2. Images of the wounded area fourteen days post-wounding acquired with the VideometerLab. Top row – control, middle row – MacroGard, botton row – 6.3 kDa oat fiber. NS – no sample.

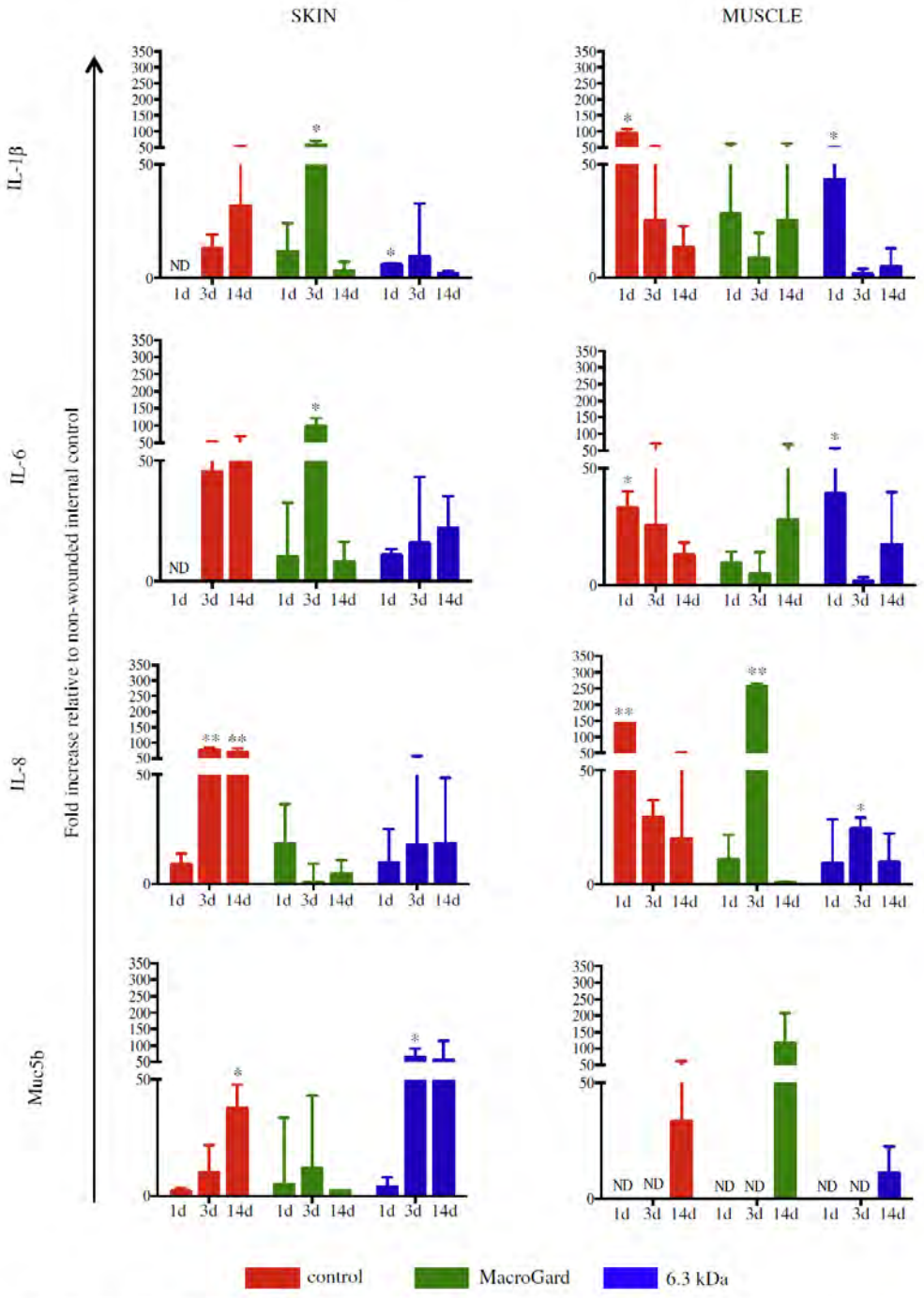


Fig. 4. Quantitative real-time PCR for mechanically wounded fish. Expression of the genes interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8) and mucin 5b (Muc5b) is shown in skin (A, C, E, F) and muscle (B, D, F, H) of common carp. Results are obtained by qPCR and expressed relative to the internal control at each time point. The data are normalized relative to 40S and analyzed using the $-2^{-\Delta\Delta Ct}$ method. Bars represent fold expression + SD relative to non-wounded side of $n = 4$ ($n = 3$ 14 days in wounded-MacroGard group). cnt – control; ND – not detected. * Depicts statistical significance between the wounded site and internal control site (*$p < 0.05$; **$p < 0.01$).

post-wounding showed a high and significant up-regulation ($p < 0.05$) (Fig. 4B).

In skin collected from the W-MG group, a significant up-regulation ($p < 0.05$) three days post-wounding was observed (Fig. 4A). No significant changes in muscle were seen (Fig. 4B).

In skin collected from the W-6.3 group, IL-1β expression was low but significant ($p < 0.05$) one day post-wounding (Fig. 4A). In muscle, high and significant up-regulation ($p < 0.05$) was observed one day post-wounding (Fig. 4B).

3.2.2. Interleukin 6 (IL-6)

In skin collected from the W-C group, IL-6 showed a tendency for up-regulation three and fourteen days post-wounding (Fig. 4C). In muscle, significant up-regulation ($p < 0.05$) was noted on day one post-wounding (Fig. 4D).

In skin collected from W-MG fish, significant IL-6 up-regulation ($p < 0.05$) was seen three days post-wounding (Fig. 4C). In musculature tissue, no significant changes were seen (Fig. 4D).

In skin collected from W-6.3 fish, a tendency for up-regulation was observed (Fig. 4C). In muscle, a high and significant up-regulation ($p < 0.05$) was detected one day post-wounding (Fig. 4D).

3.2.3. Interleukin 8 (IL-8)

In skin collected from the W-C group, three and fourteen days post-wounding, a high and significant up-regulation ($p < 0.01$) of IL-8 was observed (Fig. 4E). In muscle, a high and significant up-regulation ($p < 0.01$) was noted one day post-wounding (Fig. 4F).

In skin collected from W-MG fish, IL-8 showed a tendency to be down-regulated over time (Fig. 4E). In muscle, a high and significant up-regulation ($p < 0.01$) was detected three days post-wounding (Fig. 4F).

In skin collected from W-6.3 fish, a tendency for IL-8 up-regulation was seen fourteen days post-wounding (Fig. 4E). In muscle, significant up-regulation ($p < 0.05$) was detected three days post-wounding (Fig. 4F).

3.2.4. Mucin 5b (Muc5b)

In skin collected from W-C fish, a significant up-regulation ($p < 0.05$) of Muc5b was observed fourteen days post-wounding (Fig. 4G). In muscle samples taken fourteen days post-wounding, a tendency for up-regulation was observed (Fig. 4H).

In skin collected from the W-MG group, no significant differences in Muc5b expression were observed between wounded and non-wounded sides (Fig. 4G). In muscle, a tendency for up-regulation was noted fourteen days post-wounding (Fig. 4H).

In skin collected from the W-6.3 group, a significant up-regulation ($p < 0.05$) in Muc5b expression was observed three days post-wounding with tendency to be up-regulated fourteen days post-wounding (Fig. 4G). In muscle a tendency for up-regulation was seen fourteen days post-wounding (Fig. 4H).

3.3. Measurement of respiratory burst

Some significant differences between groups were observed (grey color), however they were inconsistent with no regular pattern. The absence of marked differences on ROS production in head kidney leukocytes was a sign of a localized immune response (refer to Table 3).

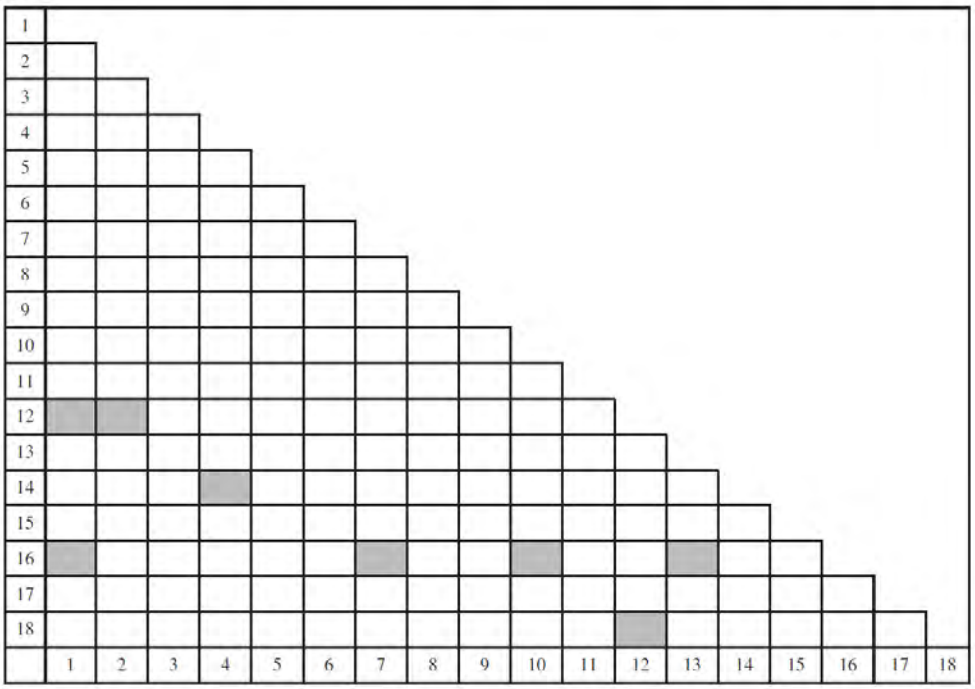
4. Discussion

Fish mucosal immunity is an extensively investigated area [54]. In contrast to terrestrial vertebrates fish skin is not keratinised and is hence metabolically active [55,56]. Following wounding, DAMPs activate mucosal immune cells as well as epithelial resident cells, e.g. in vertebrates, fibroblasts have been shown to possess immune regulation capabilities [57–59]. Several fibroblast cell lines have been established and characterised in fish, and Ingerslev et al., (2010) have reported that fish fibroblasts are susceptible to DAMPs [57,60,61]. In the present study, high up-regulation of IL-1β and IL-6 in skin samples from W-MG fish three days post-wounding could be explained by a boost of the immune-like performance from fibroblasts, elicited by the presence of DAMPs/PAMPs. Wei et al., (2002) presented the first evidence that glucans directly stimulate human fibroblasts [62]. Therefore fish fibroblasts, which could be activated in a similar way to immune competent cells, might directly stimulate the resident cells present in skin, such as epithelial, resident macrophages and lymphocytes, and amplify cytokine profiles leading to tissue regeneration. In contrast, fish Malpighian cells, regarded as the counterpart of the mammalian keratin-containing keratinocyte, which actively participate in wound healing process in fish, were shown not to react *in vitro* to yeast cells obtained from *Saccharomyces cerevisiae* [63].

Few studies have emphasized the role of goblet cells and mucus in wound healing process in mammals [11,64]. Goblet cell differentiation and function in mammalian systems are affected by mucosal immunity, and gel-forming mucins can be regulated by different cytokines or exogenous factors [2,6,33,65,66]. Van der Marel et al., (2012) have shown that Muc5b is exclusively expressed in skin and gills of common carp, therefore the Muc5b expression was expected to change in carp subjected to β-glucan bath [6]. High Muc5b up-regulation three and fourteen days post-wounding was, however, not restricted to the wound area but was a general response of the skin mucosa to the wounding. It is in agreement with work presented by van der Marel et al. (2010) where water with increased bacterial load did not induce clinical symptoms in carp, however a rapid skin mucosal response was observed even if the bacteria involved were considered to be non-pathogenic [67]. Moreover, high local inflammation and IL-1β expression could hamper Muc5b expression at the site of wounding which is in accordance with work presented by Cohan et al. (1991). This could also explain why Muc5b expression was not higher in the Macro-Gard treated group as would have been expected. However, although Muc5b in vertebrates is one of the largest gel-forming glycoprotein in the body, individual cells may differ in mucin composition [2,49,68]. Thus it cannot be excluded that β-glucan supplemented bathing induced expression of other mucins in the skin of carp which were not included in the present study. According to work presented by van der Marel et al. (2012), Muc5b was exclusively expressed in skin and gills of common carp [6]. However, in the present study, expression of Muc5b was detected in muscle samples collected fourteen days post-wounding. It was suggested by Cheng and Leblond (1974), and further by Paulus et al. (1993) and Kanter and Akpolat (2008), that columnar and goblet cells in mammals originate from a common stem cell located at the crypt base [11,69,70]. If that is the case in fish model, Muc5b detected in muscle could be an effect of goblet/mucus cell migration toward newly differentiating muscle layers.

Tissue damage and release of danger signals such as RNA/DNA or heat shock proteins influence expression of pro-inflammatory cytokines [12,27]. High levels of IL-1β, IL-6 and IL-8 cytokines one day post-wounding in muscle collected from the W-C group is likely to be the inflammatory response caused by the release of DAMPs. Interestingly, in skin samples from W-C fish, different patterns of pro-inflammatory cytokines expression has been noted. No or low IL-1β, IL-6 and IL-8 expression was detected one day post-wounding. However, gene expression was not investigated at earlier time points. It is possible that stored IL-1β, IL-6 and IL-8 have been secreted quickly (hours) in response to wounding and

Table 3
Respiratory burst significant differences within or among treatments, measured by luminol-amplified chemiluminescence of carp head kidney (HK) leukocytes. The gray color indicates the only combinations with a significant difference (p value = 0.0286). 1) non-wounded control (1 dpw), 2) non-wounded MacroGard (1 dpw), 3) non-wounded 6.3 kDa (1 dpw), 4) non-wounded control (3 dpw), 5) non-wounded MacroGard (3 dpw), 6) non-wounded 6.3 kDa (3 dpw), 7) non-wounded control (14 dpw), 8) non-wounded MacroGard (14 dpw), 9) non-wounded 6.3 kDa (14 dpw), 10) wounded-control (1 dpw), 11) wounded-MacroGard (1 dpw), 12) wounded-6.3 kDa (1 dpw), 13) wounded-control (3 dpw), 14) wounded-MacroGard (3 dpw), 15) wounded-6.3 kDa (3 dpw), 16) wounded-Control (14 dpw), 17) wounded-MacroGard (14 dpw), 18) wounded-6.3 kDa (14 dpw). .



subsequently, these stores needed to be replenished. The temporally inversed pattern of gene expression in skin/muscle tissue has been seen in both β-glucan supplemented groups, with change in expression three days post-wounding. It is consistent with work done on rodents by Wolk and Danon (1985) [25]. According to these authors, the most pronounced differences in wound closure between glucan treated and control groups took place between 48 and 96 h. All changes occurred earlier in the glucan treated group, and included proliferation and arrangement of fibroblasts as well as deposition of the collagenous matrix.

Yeast β-glucan is known as a powerful immune modulator and many studies have described its positive effect on various vertebrates [14–18,25]. Bohn and BeMiller (1995) described β-glucans as biological response modifiers with activity correlated to the branching degree and size [24]. Accordingly, no biological activity/effect was detected in mammalian models when less branched glucans with molecular weights of 5 kDa–10 kDa were used. In contrast, Tanioka et al. (2011) have shown that barley-derived β-glucan (~2 kDa) stimulates maturation of mouse dendritic cells [71]. In addition, up-regulation (~×30) of IL-6 expression in murine bone marrow cells has been seen when compared to treatment with 40–70 kDa barley β-glucan [71]. Present results show that both β-glucans, MacroGard and 6.3 kDa oat fiber, promote the wound healing process in common carp and therefore 6.3 kDa oat fiber can be considered bioactive.

To our knowledge, this is the first investigation of the biological effect of β-glucans on the wound healing process in fish. Furthermore it is the first experimental design that allows the open wounds to stay in direct and constant contact with the β-glucan supplementation during the entire trial. Many studies have described the positive effect of β-glucans on the fish immune system [13–18]. However, the mechanism by which β-glucans enhance the wound closure remains unclear [13–18]. Wolk and Danon (1985) have emphasized that application of glucans to promote wound healing in vertebrates would require an experimental model in which a wound on one side of the animal could be compared to a symmetrical wound on the other side of the same animal [25]. In our study, internal controls from non-wounded site of the wounded fish have been studied. This allowed us to eliminate external factors (e.g. temperature) and focus on obtaining more data about local and systemic response in carp during the wound closure process.

Overall, our results show that both β-glucans promote wound healing process in common carp when compared to control fish. We have concluded that bathing in β-glucans has direct positive effects on the wound closure in common carp and it can suggestible be related to high branching level due to fish being bathed in MacroGard supplemented water showed higher wound closure ratio in comparison to 6.3 kDa supplemented bath. We have shown the immunological and regenerative response following stimulation with β-glucans (PAMPs) and wounding (DAMPs) in controlled conditions without exposure to pathogens. PAMP/DAMP stimulation resulted in an inflammatory response by activating IL-1β, IL-6 and IL-8, and local differences in expression patterns depended on major stimuli: DAMPs or DAMPs/PAMPs combination. In addition, the absence of marked differences on the respiratory burst activity

in head-kidney cells supports the notion of a local immune response at the site of wound.

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Paper II

Expression of immune system-related genes during ontogeny in experimentally wounded common carp (*Cyprinus carpio*) larvae and juveniles.

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Expression of immune system-related genes during ontogeny in experimentally wounded common carp (*Cyprinus carpio*) larvae and juveniles

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Keywords: *Cyprinus carpio*, wound healing, ontogeny, gene expression, innate immune system, inflammation, immunoglobulin, IgZ.

Abbreviations: dpw, days post-wounding; dpf, days post-fertilization; 7+1 fish (and other numerical combinations separated by +) signify the age at the time of wounding (dpf) + number of days after wounding (dpw).

Highlights

- Visually wound healing is faster in larvae than in juveniles of carp.
- IgZ1 expression is upregulated as a result of wounding in early carp larvae.
- Very little local regulation of the other investigated immune system-related genes is observed in response to the wounding.

Abstract

We investigated the effect of full-thickness incisional wounding on expression of genes related to the immune system in larvae and juveniles of common carp (*Cyprinus carpio*). The wounds were inflicted by needle puncture immediately below the anterior part of the dorsal fin on days 7, 14, 28 and 49 after fertilization. We followed the local gene expression 1, 3 and 7 days after wounding by removing head and viscera before extracting RNA from the remaining part of the fish, including the wound area. In addition, we visually followed wound healing. Overall the wounds had regenerated to a point where they were microscopically indistinguishable from normal tissue by day 3 post-wounding in all but the juvenile carp wounded on day 49 post-fertilization. In these juveniles the wounded area was still visible even 7 days post-wounding. On the transcriptional level a very limited response was observed in the investigated genes as a result of the wounding. HSP70 was downregulated 1 and 3 days post-wounding in the smallest larvae. However, HSP70 was differentially expressed at different time-points in a similar manner in wounded and mock-wounded groups, thus suggesting a stress effect of the handling, which may have overshadowed some transcriptional effects of the wounding. MMP-9, TGF-β1 and IgZ1 were slightly but significantly upregulated at few time-points, while no effect of wounding was detected on the expression of IgM, C3, IL-1β and IL-6 family member M17.

1. Introduction

The objective of this study was to investigate the local transcriptional response of immune-related genes to mechanical damage during ontogeny of common carp (*Cyprinus carpio*) larvae and juveniles. Thus we aimed at examining not the timing of the first detection of hematopoietic cells and immune system-related transcripts, but the ontogeny of a functional response to tissue damage. The genes investigated were interleukin (IL)-1β, the IL-6 family member M17 (M17), heat-shock protein 70 (HSP70), transforming growth factor-β1 (TGF-β1), complement component C3 (C3), matrix metalloproteinase-9 (MMP-9) and the immunoglobulins IgM and IgZ1. In addition we visually followed the progression of wound healing.

1.1. Inflammation and wound healing

Most functional studies on the immune system in fish have focused on the response to challenge with pathogens or with pathogen-associated molecular patterns (PAMPs). However, the innate immune system not only provides protection from invading pathogens but also is intimately involved in wound healing (Eming et al., 2009), and tissue damage caused by pathogens or by sterile means have different triggers, but ultimately converges in a similar inflammatory response (Rock et al., 2010).

Wound healing proceeds either through scarless regeneration or repair with various degrees of scarring. Vertebrate fetuses and urodele amphibians are known for their ability to perfectly regenerate wounded tissue without scarring (Roy and Gatien, 2008, Roy and Lévesque, 2006). Mammalian fetal wounds exhibit less inflammation with reduced expression of IL-1β, IL-6 and TGF-β1, but with increased expression of MMP-9 and other MMPs (Dang et al., 2003, Rolfe and Grobbelaar, 2012). Larval fish have the same ability of regeneration, which persists to some extent into adulthood (Richardson et al., 2013, Yoshinari and Kawakami, 2011). Inflammation is in most situations a fundamental response to various sterile or non-sterile injuries. However, perfect regeneration usually coincides with no or limited inflammation, and excessive scarring conversely coincides with an exacerbated inflammatory response (Eming et al., 2009, Martin and Parkhurst, 2004). Nevertheless, scarless healing is possible even after substantial inflammation in zebrafish larvae and adults (Cvejic et al., 2008, Richardson et al., 2013). We followed the visual healing of the wounds and compared this to the extent of inflammation using IL-1β expression as a marker.

IL-1β is well established as a proinflammatory cytokine in all vertebrates. Its expression is highly inducible after pathogenic, chemical or mechanical stimulation (Secombes et al., 2011). Among many effects IL-1β recruits macrophages to the site of injury (Rider et al., 2011). Macrophages are important players in wound healing through phagocytosis of debris and apoptotic cells as well as secretion of growth factors and cytokines. Experimentally impairing macrophage infiltration into the wound delays healing in mammals (Eming et al., 2007, Eming et al., 2009), although zebrafish fin amputation studies show that they are not required for regeneration (Mathew et al., 2007, Niethammer et al., 2009).

The matrix metalloproteinase MMP-9 is important for leukocyte migration through the extracellular matrix (ECM) to the site of injury (Kolaczkowska et al., 2009), but also potentiates inflammation through cleavage of IL-1β, TGF-β and IL-8 (Schonbeck et al., 1998, Van den Steen et al., 2002, Yu and Stamenkovic, 2000). MMP-9 can thus be considered a proinflammatory enzyme. MMP-9 is one of the genes whose transcription is most highly upregulated in wounds, also in carp and other teleosts (Chadzinska et al., 2008, d'Alençon et al., 2010, Murakami et al., 2006, Yoong et al., 2007). MMP-9 is also likely important during ontogeny as maternal MMP-9 transcripts are deposited in the unfertilized zebrafish oocytes and autologous transcripts are detected as early as 12 hours post-fertilization in the notochord and hours later transiently in the mesoderm (Yoong et al., 2007).

1.2. Similarities between morphogenesis and tissue repair

Wound healing and developmental morphogenesis share many features (Lee et al., 2012, Martin and Parkhurst, 2004), and many of the factors that are implicated in wound healing are also important during development.

One of these factors is TGF-β, which has been considered a sensor of damage since latent precursor TGF-β complexes bound to the ECM can be activated by plasmin, gelatinases, thrombospondin-1, integrins and reactive oxygen species, which are found in areas of damage and/or inflammation (Annes et al., 2003). Activated TGF-β is pleiotropic with numerous functions including increasing the expression of ECM components by fibroblasts (Klass et al., 2009), and in mammals all three TGF-β isoforms are critical for wound healing as well as normal development (Dünker and Krieglstein, 2000, Penn et al., 2012). A few studies have investigated the effect of teleost TGF-β1 *in vitro*, and these investigations collectively suggest a pleiotropic role of TGF-β also in teleosts (Cai et al., 2010, Haddad et al., 2008, Jang et al., 1994, Yang and Zhou, 2008, Yang et al., 2012).

IL-6 and M17 are both members of the IL-6 family of cytokines. Apart from structural similarities, IL-6 family cytokines have a common signal transducing receptor protein (glycoprotein 130 (gp130)), which results in partially overlapping functions (Taga and Kishimoto, 1997). IL-6 not only mediates pro- as well as anti-inflammatory responses (Scheller et al., 2011), but has been implicated in a range of non-inflammatory functions in vertebrates (Rincon, 2012). IL-6 is implicated in wound healing, and *Il-6* knockout mice show attenuated leukocyte infiltration and delay in collagen production and overall wound healing (Lin et al., 2003). The IL-6 family member M17 was first described from common carp in 2003 (Fujiki et al., 2003) and have since been described from a number of fish species (Hanington and Belosevic, 2007, Hwang et al., 2007, Wang and Secombes, 2009). M17 has not been described from non-teleost taxa, and thus appears to be an exclusively piscine cytokine. The few investigations so far on M17 function points to an involvement in the immune and nervous systems (Fujiki et al., 2003, Hanington and Belosevic, 2007, Hwang et al., 2007, Rakus et al., 2012, Wang and Secombes, 2009). It is not known whether it plays a role in wound healing.

Although generally regarded as an important component of the innate immune system complement is also emerging as an important player in development and regeneration of the nervous system (Rutkowski et al., 2010) as well as other tissues (Ricklin et al., 2010). The complement component C3 has been shown to be important for neural crest migration in early embryogenesis in *Xenopus* and zebrafish (Carmona-Fontaine et al., 2011) as well as regeneration of limbs and lens of urodele amphibians (Del Rio-Tsonis et al., 1998, Kimura et al., 2003). The liver is the main contributor of C3 (and most other complement components) in mammals (Lange et al., 2006, Li et al., 2007, Qin and Gao, 2006), and liver is also a main contributor in adult fish (Abelseth et al., 2003, Mauri et al., 2011). However, in early life stages of some teleosts (including carp) extrahepatic C3 expression is found in a variety of tissues (Lange et al., 2004a, Lange et al., 2004b, Lange et al., 2005, Lange et al., 2006,

Løvoll et al., 2007a, Løvoll et al., 2007b, Nakao et al., 2011). The observations that complement factors are expressed at different levels in different tissues during ontogeny indicate that the complement system may also be important for non-immune-related events during teleost ontogeny.

1.3. Is antibody expression induced by wounding?

In addition to innate factors, we also followed the expression of the two immunoglobins IgM and IgZ1. Immunoglobulins are normally considered part of the adaptive immune system and are not often implicated in healing of non-infected wounds (Schäffer and Barbul, 1998). However, the so-called natural or innate antibodies have a broad binding spectrum and also bind self-antigens. They are involved in removal of aberrant and apoptotic cells (Baumgarth, 2013, Chow et al., 1999, Kim et al., 2002, Lutz, 2007, Whyte, 2007) and antibodies enhance wound healing in mice (Nishio et al., 2009).

In mammals innate antibodies are produced by B-1 cells, which are present from very early in development (Boes, 2000, Whyte, 2007). B-1 cells are found in the periphery as well as lymphoid organs (Baumgarth, 2013), are self-replenishing (Ghosn et al., 2012, Kantor et al., 1995), share a developmental kinship with myeloid cells (Gao et al., 2012, Popi et al., 2012), migrate to sites of inflammation (Popi et al., 2004) and are phagocytic (Gao et al., 2012, Parra et al., 2012). The latter is also a feature of B-cells from other vertebrate taxa, including fish (Li et al., 2006, Nakashima et al., 2012, Øverland et al., 2010, Zimmerman et al., 2010). Murine B-1 cells participate in wound healing where they have an anti-inflammatory effect (Oliveira et al., 2010), but this has not been demonstrated for fish.

The carp adaptive immune system develops at a very early age compared to many other fish species (Tatner, 1996), and immunoglobulin transcripts are present already from the embryonic stage (Ryo et al., 2010). Carp are considered embryos until the end of yolk absorption and start of first feeding at approximately 5 days post-fertilization (dpf) (Huttenhuis, 2005), while the larval to juvenile transition (metamorphosis) takes place at 20-25mm standard length (~20-30dpf) (Vilizzi and Walker, 1999).

IgM and IgT/IgZ are usually described as systemic and mucosal immunoglobulins, respectively (Zhang et al., 2010). The mucosal immune system is important in adult fish, but could be speculated to be even more important in larvae as the relative surface area is larger (Vilizzi and Walker, 1999). In fact, at the time of discovery IgZ was already found to be expressed early in development and IgZ^+ B-cells were speculated to be equivalent to mammalian B-1 cells (Danilova et al., 2005) despite the fact that these B-1 cells produce immunoglobulin of mainly the IgM class (Baumgarth, 2013). We were interested in investigating if antibodies were involved in wound healing, and if so; whether IgZ1 could be relatively more important than IgM in larvae compared to juveniles due to the higher surface to volume ratio in the former.

In summary, we wanted to investigate the developmental and comparative response of carp larvae and juveniles to wounding. We did this by following the transcriptional response of select immune-related genes as well as the visual healing of the inflicted wounds.

2. Materials and methods

2.1. Fish

Fertilized eggs from the R3xR8 strain of common carp (*Cyprinus carpio* L.) were moved from the facilities at Wageningen University in The Netherlands to the facilities at The Technical University of Denmark where the majority hatched the day after arrival (2 days post-fertilization), and the remainder one day later. They were kept in 30L aquaria at 25°C and 12:12 light:dark photoperiod at all times. The fish were fed *Artemia* nauplii (including unhatched eggs) initially, but this diet was supplemented with dry granulate feed from day 36 post-fertilization.

2.2. Experimental setup, experimental wounding and sampling

In order to standardize the stimulus for a subject that changes substantially in morphometrics as well as size during the investigated period we decided to use a hypodermic needle to inflict an incisional wound. The tapering of the needle to some extent addressed the change in size.

On day 7 post-fertilization some of the fish were brought to the lab. They were moved individually with a plastic pipette to anaesthesia (tricaine methanesulfonate (MS-222); Sigma, Broendby, Denmark). Once anaesthetized, the fish was moved to a small plastic dish under a dissection microscope. Here it was experimentally wounded by complete piercing from left to right with a 25 G needle in an area just below the anterior part of the dorsal fin. A digital image was acquired after which the fish was placed in an aquarium with freshwater. Every second fish underwent a mock procedure in which only the piercing with the needle was left out. In addition, 7 unhandled control fish were killed in an overdose of MS-222, measured and weighed and placed in an eppendorf tube containing RNAlater™ (Ambion, Austin, Texas) on ice. Wounded and mock-wounded fish were kept in separate aquaria at all times. On days 1, 3 and 7 post-wounding seven mock-wounded fish and seven wounded fish were brought to the lab. One by one they were placed in anaesthesia, but this time in a lethal dose of MS-222. Each fish was then placed under the dissection microscope and an image acquired in which total length was measured. The fish was then weighed and finally placed in an eppendorf tube containing RNAlater™ on ice. On every sampling day additional close-up microscopic images of the wounds were acquired of one or two representative fish that were wounded in surplus. After all sampling of the day had been performed samples were placed at 4°C for 24h, and then moved to -20°C until further processing.

This procedure was copied for fish at days 14, 28 and 49 post-fertilization. The only difference was that the larger fish were handled with a small spoon when anesthetized and a small net when not. No fish died as a result of the wounding. The experiment was conducted according to Danish legislation and by scientists accredited by the Federation of Laboratory Animal Science Associations (FELASA).

Condition factor was calculated from length and weight data and was used as a simplified approximation of the volume:surface relationship.

2.3. RNA extraction

For the extraction of mRNA, the fish were moved from the RNAlater™ and placed under a dissection microscope where the head and viscera were removed with a scalpel and discarded (see fig. 1). RNA was then extracted from the remaining part of the fish using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Broendby, Denmark) and following the manufacturer's instructions. Tissue was disrupted by sonication in lysis buffer (using a Sonics Vibracell sonicator, Sweden, fitted with a model CV18 probe and set to 20s, pulse 2, amplitude 70 %). The RNA was eluted in a final volume of 30µl.

The extracted RNA was treated with DNase 1, Amplification Grade (AMPD1-1KT, Sigma-Aldrich, Broendby, Denmark) according to manufacturer's instructions to remove contaminating genomic DNA. Immediately hereafter RNA quantity and purity (OD_{260}:OD_{280} ratio) was measured on the NanoDrop 1000 (Thermo Fisher Scientific, Germany). All OD_{260}:OD_{280} values were between 1.87 and 2.24.

Figure 1. Prior to extraction of RNA from the samples, head and viscera were removed by cutting with a scalpel at the two lines illustrated in this figure. Only the remaining dorsocaudal part was used for further analysis, except in a few cases as described in the article. A one day old wound can be clearly seen below the dorsal fin of this 15dpf carp larva.

2.4. Reverse transcription

The RNA was reverse transcribed with TaqMan® Reverse Transcription reagents (N808-0234, Applied Biosystems, Foster City, USA) primed with random hexamer primers (2µl 10x RT buffer, 4.4

Gene		Primer sequence (5'–>3')	Amplicon size (bp)	GenBank acc. no.	Reference
40S	Forward	GTTGAAGGAAGTGGCAAGGA	146	AB012087	González et al., 2007a
	Reverse	AGAATACGGCCTCTGATGGA			
IL-1β	Forward	AAGGAGGCCAGTGGCTCTGT	69	AJ245635	González et al., 2007a
	Reverse	CCTGAAGAAGAGGAGGCTGTCA			
M17	Forward	CCGCACATGAAGACAGTGAT	150	AY102632	Przybylska et al., 2013
	Reverse	GGGTATATTTGGCTGCAGGA			
HSP70	Forward	GGCAGAAGGTGACAAATGCA	No data	AY120894	Stolte et al., 2009
	Reverse	TGGGCTCGTTGATGTTCTCA			
TGF-β1	Forward	ACGTTTATTCCCAACCAAA	97	AF136947	Huttenhuis et al., 2006a
	Reverse	GAAATCCTTGCTCTGCCTCA			
IgM	Forward	CACAAGGCGGGAAATGAAGA	No data	No data	Ryo et al., 2010
	Reverse	GGAGGCACTATATGAACAGCA			
IgZ1	Forward	TACAAACAGCATGAGCCAGCT	No data	No data	Ryo et al., 2010
	Reverse	ACTCCCTGGTGTGTGACCTC			
C3	Forward	GTCGGTCCTGGACTGTCTCT	113	AB016211-AB016215	González et al., 2006b
	Reverse	AGTGCACTGCTTCTCCTGCT			
MMP-9	Forward	ATGGGAAAGATGGACTGCT	No data	AB057407	Chadzinska et al., 2008
	Reverse	TCAAACAGGAAGGGGAAGT			

Table 1. Data for primers used for real-time qPCR.

µl 25mM $MgCl_2$, 4µl dNTP mix, 1µl Random Hexamer Primers, 0.4µl RNase inhibitor, 1.25µl Multiscribe Reverse Transcriptase and 400ng RNA diluted in 6.95µl RNase free water; final reaction volume 20µl). Samples were prepared in 0.2µl tubes which were placed in a thermocycler (Veriti,

Applied Biosystems, Foster City, USA) and run for 10min at 25°C, 60min at 37°C and finally 5min at 95°C. The samples were then diluted 1:10 in MilliQ water and stored at -20°C.

2.5. Gene expression analysis

Gene expression was quantified with real-time quantitative polymerase chain reaction (qPCR) using SYBR® Green on a Stratagene MX3000P™ thermocytable 2cler. Primer specificity was confirmed by a melt curve analysis. The investigated genes were IL-1β (the IL-1β1 isoform, not the IL-1β2 isoform), M17, HSP70, TGF-β1, C3 (the primer set was designed to amplify all five isoforms present in carp), MMP-9, IgM and IgZ1. 40S ribosomal protein S11 (40S) was used as an internal reference gene. 40S has previously been demonstrated to be stably expressed in developing carp larvae as well as in juvenile carp subjected to damage (Gonzalez et al., 2007, Huttenhuis et al., 2005). Primer sequences, amplicon lengths and GenBank accession numbers are shown in table 1. All genes for each sample were investigated in the same run, including reference gene and non-template control. A master mix was prepared from SYBR® Green JumpStart™ *Taq* ReadyMix™ kit (S4438, Sigma-Aldrich, Broendby, Denmark) using the following relative volumes (shown as final volume per well): 12.5µL SYBR® Green JumpStart™ *Taq* ReadyMix™, 0.5µL ROX reference dye (deleted 10X) and 9.0µL DEPC-treated water (Sigma-Aldrich, Broendby, Denmark). This mix was aliquoted into tubes corresponding to the number of cDNA samples tested on the given day and diluted cDNA was added (corresponding to 1.0µL per well). The mix was transferred to a 96-well plate and forward and reverse primers were added (1.0µL of each per well). The cycling conditions were 2min at 94°C followed by 42 cycles of 15s at 94°C and 1min at 60°C. Primer specificity was subsequently confirmed by a melt curve analysis, which was initiated with a denaturing step for 1min at 94°C followed by a gradual increase from 60°C to 94°C with fluorescence measurements at short intervals.

2.6. Statistical analysis

Due to few replicates a normal distribution could not be assumed, thus the Mann-Whitney U-test was used to calculate gene expression differences between wounded and control fish as well as between time-points. Fold difference between the wounded and unwounded groups was calculated with the $2^{-\Delta\Delta Ct}$ method. Differences were regarded as significant at $P<0.05$. In the cases were no Ct values were obtained these samples were left out of the analysis. The software Prism version 4.03 (Graph Pad, La Jolla, CA, USA) was used for the statistical analysis and the graphic representation of the expression data and the condition factor data.

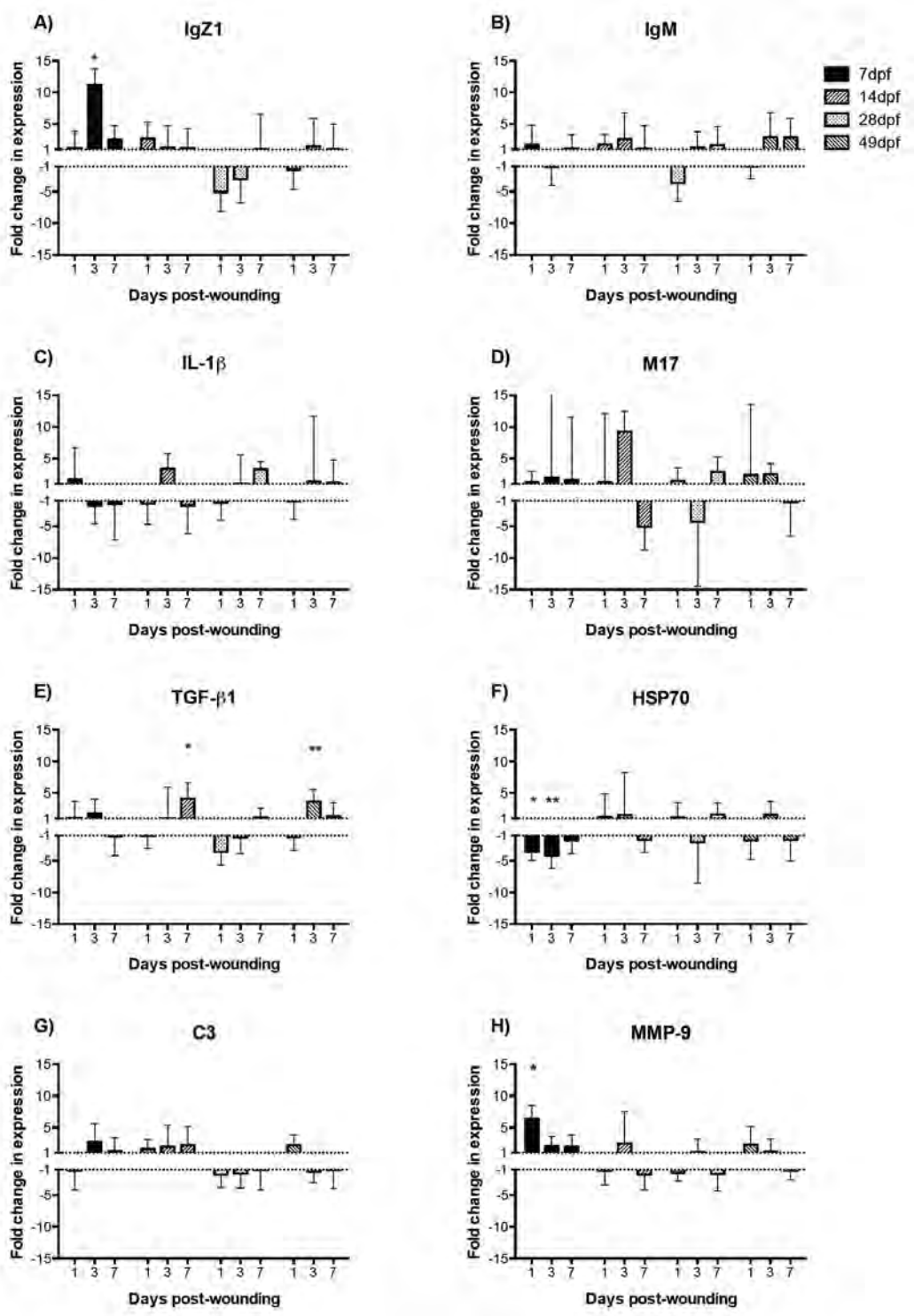


Figure 2. Fold differences ($2^{-\Delta\Delta Ct}$) in expression of the investigated genes of wounded versus unwounded groups normalized to 40S ribosomal RNA. Data is shown as the mean with standard deviation. Asterisks denote statistic significance (Mann-Whitney U test) between wounded and unwounded groups at the given time-point (*=P<0.05, **=P<0.01). dpf=days post-fertilization. The number of replicates (n) are 4≤n≤7 for the different groups, and specifically number of replicates (n) for the statistically significant results are: IgZ1 day 7+3, n=5; TGF-β1 day 14+7, n=7; TGF-β1 day 49+3, n=5; HSP70 day 7+1 and 7+3, n=5; MMP9 day 7+1, n=5.

3. Results and discussion

3.1. All investigated genes were expressed at all investigated time-points, but wounding had little effect on gene expression

All investigated genes were expressed at all investigated developmental stages, whether wounded or not. Larvae wounded 5dpf had a significantly higher expression of MMP-9 ($P<0.05$) and a lower expression of HSP70 ($P<0.05$) 1 day post-wounding (dpw) compared to mock-wounded, and at 3dpw HSP70 expression was still lower ($P<0.01$) while IgZ1 was now significantly upregulated ($P<0.05$). TGF-β expression was significantly higher in 14+7 (age at time of wounding in dpf+dpw) larvae ($P<0.05$) and 47+3 juveniles ($P<0.01$; fig. 2).

Since IL-1β is a very inducible, prototypic marker of inflammation, the lack of significant upregulation indicates either a limited or lacking inflammatory response to the stimuli or a fast resolution preceding the first sample point at 1dpw.

In zebrafish it is possible to follow myeloid cell migration *in vivo*. In 2dpf zebrafish embryos inflammatory myeloid cells starts infiltrating a wound within minutes, numbers peak after 3-6 hours and most are gone already after 24h (Lévesque et al., 2013). In adult zebrafish wounds myeloid cell numbers peak at 12h and gradually decline until pre-wounding levels are reached 3dpw (Richardson et al., 2013), and in adult carp granulocyte numbers peak 12-24h post-wounding and macrophage numbers 4dpw in full-thickness incisional wounds (Iger and Abraham, 1990). We thus expected minimal detectable inflammation in early larvae, but substantial inflammation until at least day 1 post-wounding in juveniles. However, this was not the case.

Similarly to IL-1β MMP-9 is highly expressed in adult wounds, but was only significantly upregulated at one time-point in this experiment. In zebrafish larval fin amputation sites MMP-9 is still strongly expressed 4dpw (Yoshinari et al., 2009), so the limited wound-induction of MMP-9 was unexpected, also considering the elevated MMP-9 expression observed in mammalian fetal macrophages and wounds (Dreymueller et al., 2013, Rolfe and Grobbelaar, 2012). However, amputation is a more severe injury than an incisional wound, and it is possible that excisional wounds would have resulted in a stronger inflammatory response.

At least in mice, upregulation of C3 expression also depends on an inflammatory response. PU.1 null mice (which are deficient in myeloid cells) have a similar constitutive expression of C3 in intact skin as wild type mice, but a severely reduced induction following wounding (Cooper et al., 2004). Given the absence of inflammation, it is not surprising that no effect of wounding on C3 expression was observed.

In the case of M17, expression levels were quite low and variable, and it is not possible to draw any clear conclusions on the involvement of M17 in wound healing based on this experiment.

The lack of significant regulation of inflammation-related genes may be explained by similarities between morphogenesis and tissue repair. The effects at the wound site may to some extent be mirrored by events that are already taking place in the fish.

3.2. Handling effects may have overshadowed wound effects

HSP70 is considered a stress inducible HSP with no or limited constitutive expression (Krone et al., 1997), and HSP70 is also stress inducible in fish (Deane and Woo, 2011).

There was apparently an effect of the experimental procedure notwithstanding the actual wounding, since repetitive patterns were observed in the expression levels of HSP70 after mock wounding. In fact these temporal differences were much more pronounced than the wound effects (fig. 3F). The juveniles appear to be less affected than the larvae, and there is no significant effect of handling nor wounding on HSP70 expression at this developmental stage.

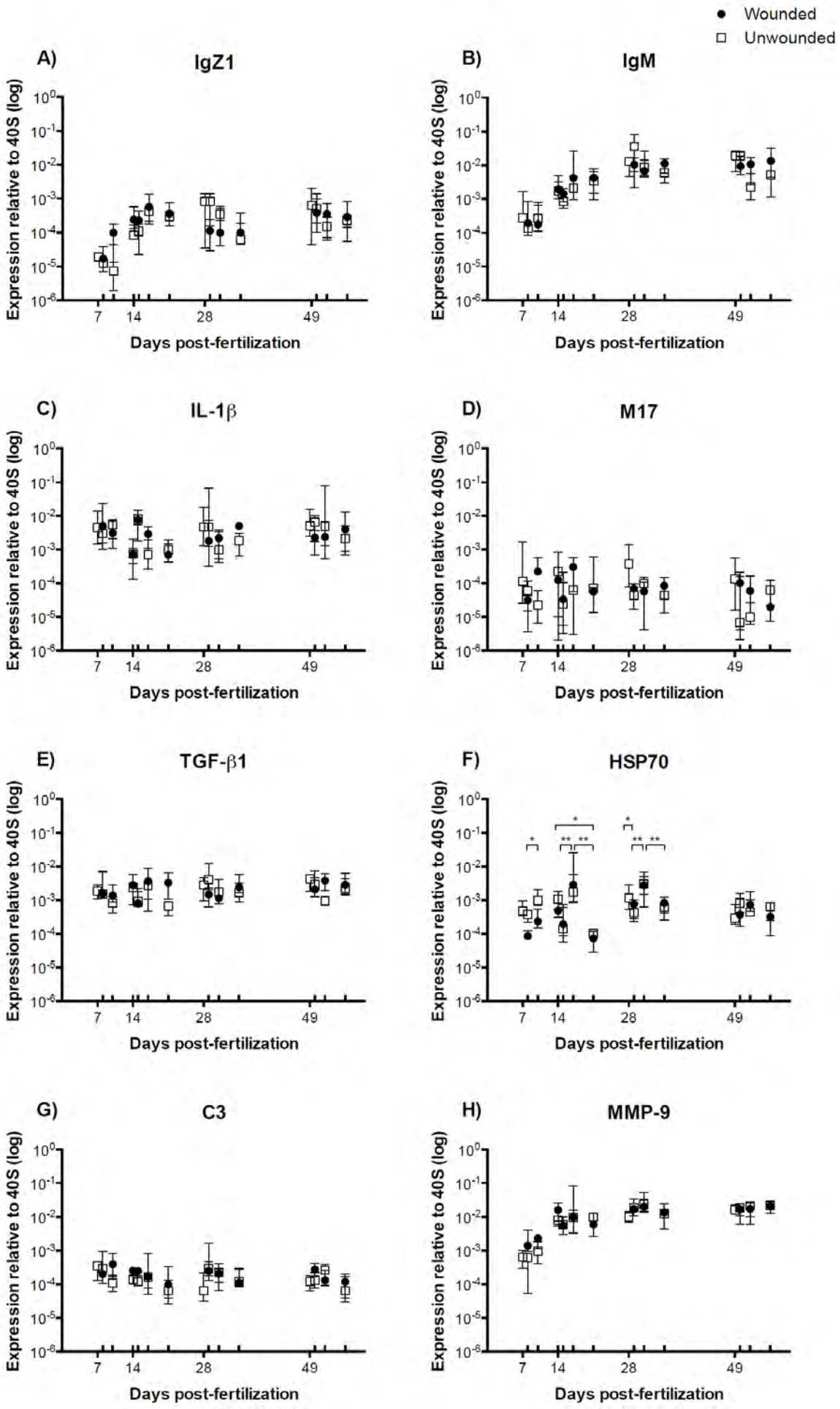
A number of studies have looked at the involvement of HSPs in wound healing and during ontogeny. These studies generally report constitutive expression of HSP70, but levels vary with species, cell type and tissue as well as developmental stage (Campos et al., 2013, Deane and Woo, 2011, Dietz and Somero, 1993, Fuzzen et al., 2011, Oyarbide et al., 2012, Yeh and Hsu, 2000, Yeh and Hsu, 2002). In zebrafish HSP70 is involved in angiogenesis and lens development, as well as in optic nerve regeneration following damage (Blechinger et al., 2002, Bruns et al., 2012, Evans et al., 2007, Nagashima et al., 2011). The induction of HSP70 at damaged sites is an ancient response found in invertebrates as well as vertebrates (Evans et al., 2007, Laplante et al., 1998, McMurtry et al., 1999, Zampell et al., 2011).

Since a sudden temperature shift is a strong inducer of HSP70, heat stress could be speculated to be caused by the supposed elevated temperature under the dissection microscope (although this was not measured). However, gene expression of unhandled controls was only investigated on the day of wounding, thus it cannot be completely ruled out that the observed differences are due to natural developmental events.

3.3. Basal expression of IgM, IgZ1 and MMP-9 increase during the larval stage, but levels off before the end of metamorphosis

Most of the investigated genes were expressed at comparable levels at all times 7-56dpf (fig. 3). However, in the case of MMP-9, IgZ1 and IgM there was an initial gradual increase in expression (figure 3A, 3B, 3H). This increase was approximately 30-50 fold for all three genes, but the kinetics differed with MMP-9 and IgZ1 expression reaching a plateau around 14-15dpf, and IgM expression increasing until circa 28dpf. The mRNA levels were consistently greater for IgM than for IgZ1.

Keratinocytes and myeloid cells are the main source of MMP-9 in humans (Van den Steen et al., 2002). This may be similar in fish as adult teleost MMP-9 expression is highest in leukocyte-rich organs (Castillo-Briceño et al., 2010, Chadzinska et al., 2008, Yoong et al., 2007) and in zebrafish and


Wounded
Unwounded
A)
IgZ1
B)
IgM
C)
IL-1β
D)
M17
E)
TGF-β1
F)
HSP70
G)
C3
H)
MMP-9
Expression relative to 40S (log)
Days post-fertilization
7
14
28
49

←**Figure 3.** Expression of the investigated genes during ontogeny shown as -1/ΔCt values relative to 40S ribosomal RNA. Experimental groups of fish are plotted as the mean with standard error. Closed circles are wounded fish, open squares are unwounded control fish. Lower ticks on the X-axis show the day of the wounding, upper ticks are the days (1, 3 and 7) after each of the days of wounding. Please note log scale of the Y-axis. Figure 3F additionally shows significant differences in HSP70 expression in control fish (statistical data for wounded fish not shown) on different days after mock-wounding (Mann-Whitney U test, *=P<0.05, **=P<0.01). The temporal expression differences of HSP70 are far greater than the wound-induced differences (*cf.* fig. 2F). HSP70 is stress inducible. Thus, there is a likely stress effect of the wounding procedure notwithstanding the actual wounding, although an unhandled control was not available to completely rule out if the differences were mere natural fluctuations during ontogeny.

fat snook (*Centropomus parallelus*) neutrophils appear to be the main MMP-9 expressing cell type (da Silva et al., 2011, Yoong et al., 2007). However, in zebrafish fin regeneration studies expression of MMP-9 induced proximal to the wound edge is mostly from cells that are not of myeloid origin, but are more likely epidermal/mesodermal (Yoshinari et al., 2009). In the present experiment MMP-9 was only wound-induced in 7+1 larvae (fig. 2H), but the ontogenetic increase in expression may reflect a developmental increase in myelopoiesis.

Similarly, the increase in IgZ1 and IgM transcript levels could reflect expansion of the IgZ^+ and IgM^+ lineages of B cells. However, it is surprising that we obtain very similar results to those reported for IgZ1 and IgM by Ryo et al. (Ryo et al., 2010) for whole larvae. Removing the head and viscera – and thereby important primary and secondary lymphoid organs such as thymus, kidney and spleen – was expected to result in lower expression levels in the remainder of the fish. Thus, these results demonstrate an early peripheral expression of immunoglobulins.

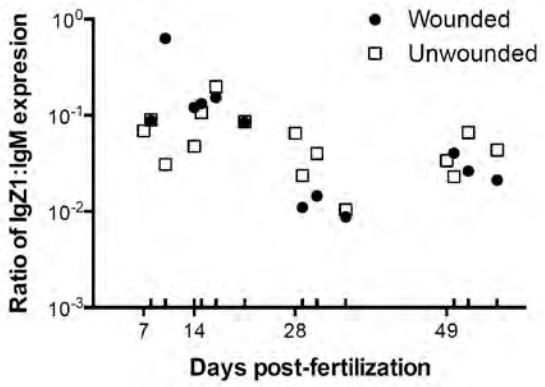


Figure 4. Ratio of IgZ1 to IgM expression. Closed circles are wounded fish, open squares are unwounded control fish. Values are shown as the average ΔCt values of IgZ1/IgM. Note log scale of Y-axis.

The IgZ1 to IgM ratio increases until metamorphosis after which it decreases rapidly and remains relatively constant (fig. 4). IgZ1 thus seems to be relatively more important in larvae than in juveniles.

Before metamorphosis growth is allometric with respect to most traits, but from onset of the juvenile period growth is largely isometric (Vilizzi and Walker, 1999). One of the traits that change allometrically during the larval period is the volume to surface ratio. Condition factor is used here as a crude estimate of volume to surface ratio, and hence systemic to skin mucosal ratio. The condition factor of the carp increases from close to 0.5 at 7dpf to more than 1.5 at 56dpf (fig. 5), indicating relatively more surface to volume in larvae. Thus a higher IgZ1 to IgM expression ratio concurs with a higher surface to volume ratio.

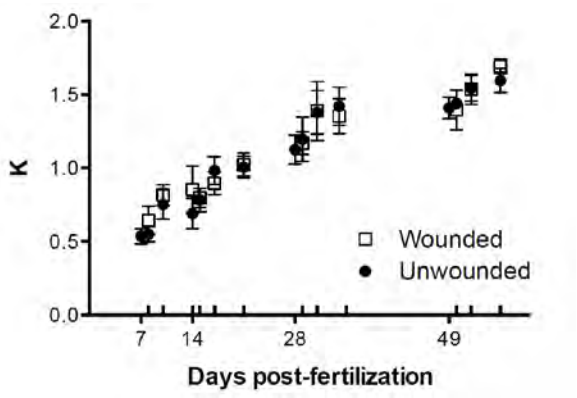


Figure 5. Condition factor of the carp from 7dpf to 56dpf. The condition factor changes from close to 0.5 at 7dpf to more than 1.5 at 56dpf. Compare to the morphometrics of the carp in fig. 7. An interesting repetitive feature is the stepwise increase in condition factor between 1 and 3dpw.

3.4. Visual wound healing

All experimental fish were photographed under the dissection microscope on the day of sampling before being put into RNAlater™. In addition, on each sampling day one or two representative fish, which were not used for gene expression studies, were used for higher resolution images under a conventional light microscope (fig. 6).

The wound site was visible 1dpw in all investigated carp, although in 7+1 larvae this usually manifested itself as no more than a vaguely opaque area. In larger fish there was still varying amounts of blood visible 1dpw. For the carp wounded 7, 14 and 28dpf the wound site was not visually distinguishable from normal tissue in most carp at 3dpw, and in all carp at 7dpw (table 2). However, the wound was still visible even 7dpw in all but one of the carp wounded 49dpf. In these juveniles there was still a rosy hue at the wound site 3dpw, possibly due to unresolved bleeding. This was not present 7dpw. At 3 and 7dpw the wound area was pale and devoid of large melanophores, but with numerous smaller dark spots. In the cyprinid Rohu (*Labeo rohita*) similar smaller spots at wound sites are also reported to be melanophores (Rai et al., 2012). The absence of (large) melanophores at the wound site correlates well with the failure to detect inflammation through upregulated expression of IL-1β, since wound hyperpigmentation depends on an inflammatory response in zebrafish (Lévesque et al., 2013).

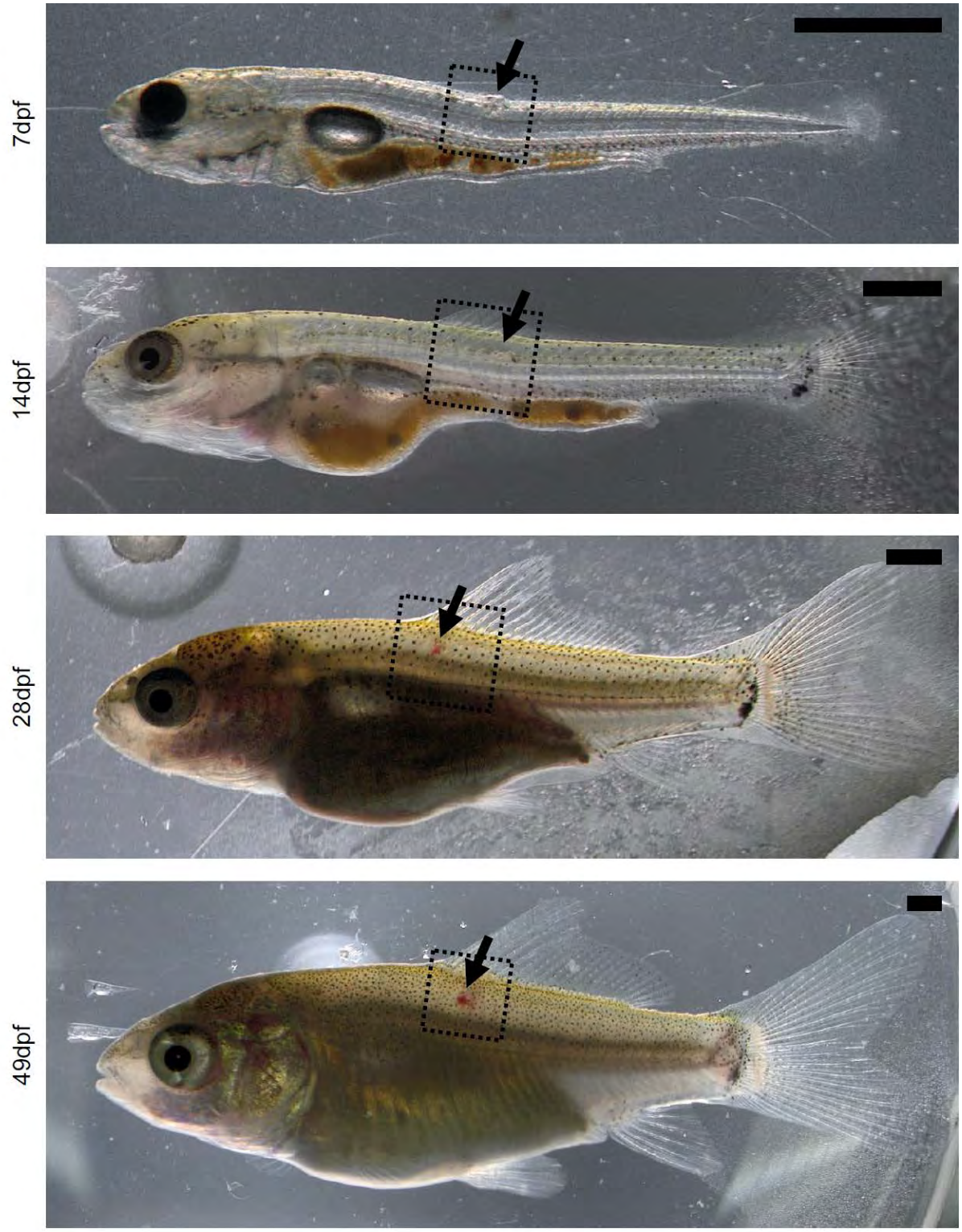
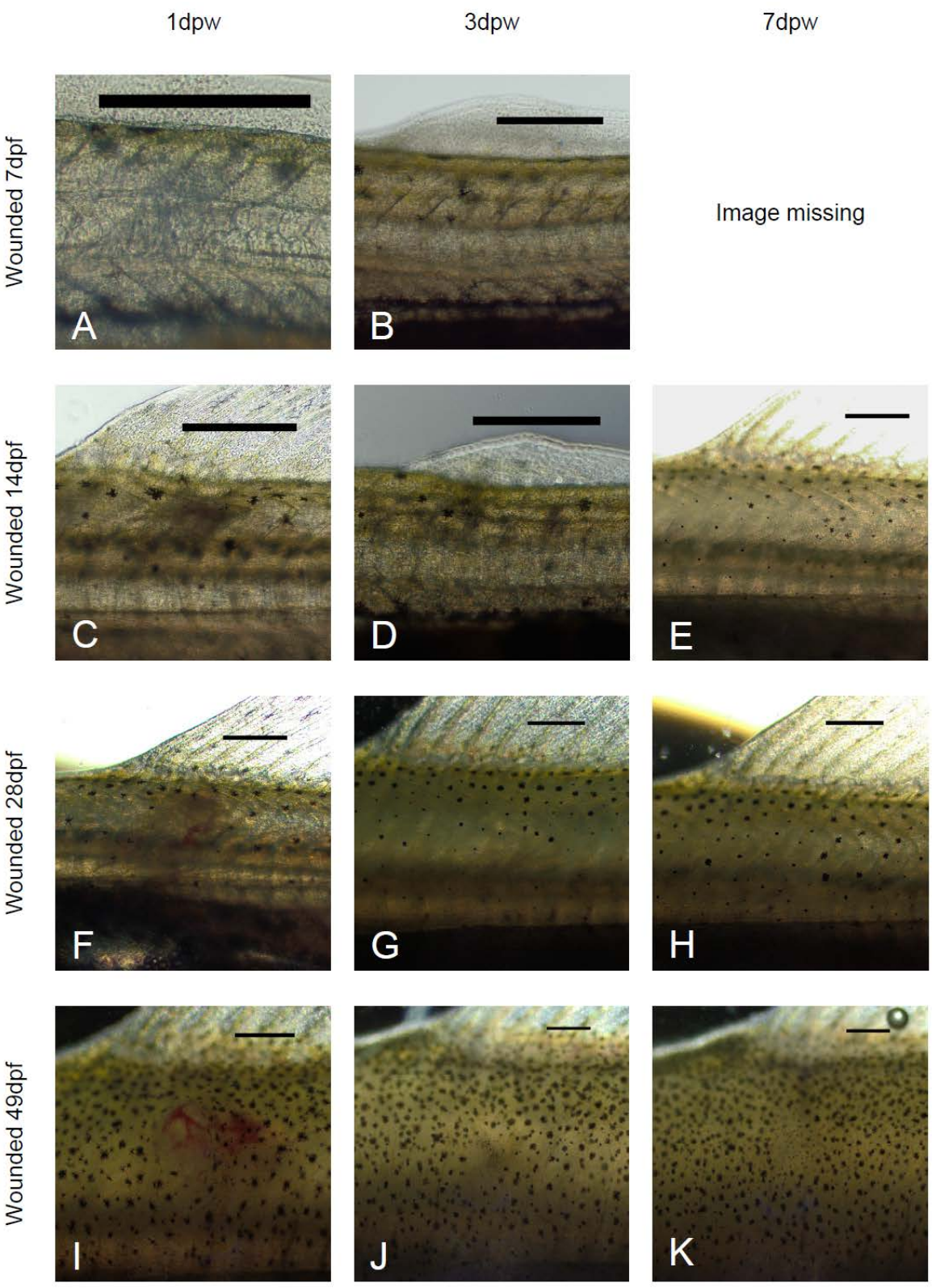


Figure 6. Images showing representative carp immediately after wounding 7, 14, 28 and 49 days post-fertilization (dpf). Arrows point to the wound site. Note that there is so little muscle tissue in the smallest larvae that the needle puncture also affects the notochord, which introduces a temporary small bend in the larvae. The notochord can also be seen at higher magnification at the centre of fig. 8A, with the angled lines of myosepta visible at both sides. Dotted squares outline the approximate location of the images in fig. 8. Black bars=1mm.


1dpw
3dpw
7dpw
Wounded 7dpf
Wounded 14dpf
Wounded 28dpf
Wounded 49dpf
Image missing
A
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←**Figure 7.** Images showing the progression of wound healing in carp larvae and juveniles. The wound was inflicted with a 25G hypodermic needle 7 days post-fertilization (dpf; A, B), 14dpf (C, D, E), 28dpf (F, G, H) or 49dpf (I, J, K), and the images show the wound site 1, 3 and 7 days post-wounding (dpw) in a representative fish[a] that was wounded at the same time as the fish that were processed for gene expression. For all carp the wound site was still visible 1dpw. At 3dpw the wound had generally visually healed in all but the 49+3 juveniles. There was no visual evidence of the wound in any of the 7+7 and 14+7 larvae and 28+7 young juveniles. However, in all but one of the 49+7 juveniles the wound site was still visible and there were no large melanophores at the wound site. The length of the black bar in the upper right corner of each image is 500µm.

[a] It should be noted that the fish in image D (14+3) was not a good representative for its group as it was smaller and lagging in development as can be seen by the larval fin fold with no apparent fin rays.

	1dpw	3dpw	7dpw
7dpf	7	0	0
14dpf	7	1	0
28dpf	7	2	0
49dpf	7	7	6

Table 2. Visual healing of wounds. The table shows the number of fish (out of 7) in which the wound site was visible. dpf = day of wounding post-fertilization; dpw = day post-wounding. The darker the background, the more individuals with completely regenerated wounds. Black=wounds healed in all fish. White=wounds visible on all fish.

Conclusion

In conclusion, a very limited transcriptional response to wounding was observed. Natural events in the rapidly transforming carp larvae may mirror and mask the wound-induced tissue rearrangement and gene regulation.

In this experiment, HSP70 expression data indicate possible handling stress, and this may additionally have confounded some of the wound effects.

The immunoglobulins IgM and IgZ1 were expressed in the periphery from the earliest investigated time-points, and IgZ1 was significantly upregulated as a result of the wounding in the smallest larvae.

Wounds regenerate faster in larvae than in juveniles. In larvae the wound had generally regenerated by day 3 post-wounding, whereas the wound was still visible after 7 days in juveniles.

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Paper III

Long-term investigation of healing of full-thickness cutaneous excisional wounds in rainbow trout (*Oncorhynchus mykiss*)

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Long-term investigation of healing of full-thickness cutaneous excisional wounds in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

We followed the healing of full-thickness excisional cutaneous wounds over the course of 100 days in rainbow trout (*Oncorhynchus mykiss*) subjected to a bathing in a β-glucan product (MacroGard). At regular intervals (1, 3, 7, 14, 38 and 100 days post-wounding) we sampled muscle at the wound edge for gene expression and acquired digital images for analysis of visual wound closure kinetics. The investigated genes have all previously been investigated in relation to wound healing, but only few in relation to wound healing in fish. The selected genes were interleukin (IL)-1β, IL-6, transforming growth factor (TGF)-β1 and -3, matrix metalloproteinase (MMP)9 and -13, inducible Nitric Oxide Synthase (iNOS), fibronectin (FN), tenscin-C (TN-C), prolyl 4-hydroxylase α1-chain (P4Hα1), lysyl oxidase (LOX) and collagen type I α1-chain (ColIα1). The genes were chosen as representatives of different wound healing stages such as inflammation, granulation and mature matrix formation, and matrix remodeling. In addition we included cell specific marker genes for thrombocytes (CD41) and a potential marker for M2 polarized macrophages (CD163).

We found no effect of β-glucan treatment on wound closure and very limited effect on gene expression. In both groups wound healing progressed very slowly. The inflammation phase lasted more than 14 days, and the genes related to production and remodeling of new matrix exhibited a delayed but prolonged upregulation starting 7-14 days post-wounding and lasting until at least 100 days post-wounding. The gene expression patterns indicate limited capacity for muscle regeneration in rainbow trout, and texture analyses confirm that wounds heal with fibrosis. CD41 expression was significantly elevated in the wounds until 38 days post-wounding indicating persistence of thrombocytes in the wound and possible active lasting involvement in wound healing beyond hemostasis. CD163 only showed borderline significant changes in expression until day 100 post-wounding when CD163 was significantly upregulated. This is consistent with the expected timing of presence of “wound healing” M2c macrophages. CD163 may thus potentially prove a valuable marker of M2 macrophages – or a subset hereof – in fish.

1. Introduction

Healing of damaged muscle tissue has been the subject of only few investigations at the molecular level in fish. Instead, fin amputation is the method of choice for piscine regeneration studies and zebrafish (*Danio rerio*) the species of choice. These studies show that the zebrafish fin heals quickly and with almost perfect regeneration (Yoshinari and Kawakami, 2011). However, fish fins do not contain muscle. Healing of deep cutaneous wounds was recently described from zebrafish, and in this study muscle did not regenerate to the same extent as skin and contained additional adipocytes (Richardson et al., 2013). The zebrafish is a small, warm water species with a determinate growth pattern, and thus not a very good model for fish such as salmonids, which are mostly natural inhabitants of fast flowing cold-water rivers, with the possibility of adopting an anadromous strategy. Ingerslev et al (Ingerslev et al., 2010) investigated the effect of incisional needle damage on gene expression in rainbow trout muscle. Here we follow the expression of a different set of genes day 1-100 post-wounding in an excisional wound model. Several of the selected genes have not yet been investigated in salmonids.

We recently reported that PAMP bathing resulted in accelerated wound contraction in common carp (*Cyprinus carpio*) (Przybylska-Diaz et al., 2013). The immune strategies deployed by these two species have likely evolved as a consequence of their very different use of habitat and thus their natural exposure level to PAMPs. Here we investigate the effect of PAMP bathing on wound healing in rainbow trout.

Wound healing consists of several partially temporally overlapping events. Briefly described, these include hemostasis, inflammation, granulation tissue formation, angiogenesis, re-epithelialization, wound contraction, and maturation and remodeling of the extracellular matrix.

We used image analysis to investigate differences in wound closure kinetics and real-time quantitative PCR (RT-qPCR) to investigate gene expression kinetics in the injured muscle over the course of 100 days. The investigated genes were chosen as representatives of some of the phases and cells involved in wound healing, and the expression of several of these genes have not yet been investigated in rainbow trout. These included cytokines and proteases mainly expressed during the inflammatory phase (IL-1β, IL-6, TGF-β1, TGF-β3, MMP9 and MMP13), genes involved in rebuilding of the extracellular matrix (ECM) (FN, TN-C, ColIα1, LOX and P4Hα1) as well as CD41, iNOS and CD163. The latter three are mammalian markers for platelets and M1 and M2 polarized macrophages respectively. While CD41 in zebrafish (*Danio rerio*) is reported to mirror the cellular expression pattern of mammals (Ma et al., 2011), markers of macrophage subsets are not very established in fish (Forlenza et al., 2011)

2. Materials and methods

2.1. Animals and experimental facilities

The rainbow trout used in this study were kept from small fingerlings at the experimental facilities in aerated 600L freshwater tanks. At the start of the experiment they had a weight of 112.2±20.9g and a fork length of 20.6±1.2cm. The experimental facilities consisted of a modified 30' container mounted with air condition, water and lighting in addition to tanks and a water filtration system. The container was not isolated. The experiment was initiated in early December 2010, and the air conditioner was not able to keep the temperature constant at the desired 12°C during the unusually cold winter. In addition, the tanks were regularly supplemented by a slow flow of fresh tap water (which was typically 6-7°C). The water temperature in the tanks thus varied considerably as a result of ambient air temperature changes and water change. Water temperature in the tanks was measured manually immediately before and after water change with 0.5°C accuracy. The data plotted in figure 1 thus represents the temperature extremes experienced by the fish. The light:dark cycle was 12:12h. Throughout the experimental period the fish were hand fed a standard commercial feed (EFICO Enviro 920, BioMar A/S, Brande, Denmark) at 0.8% body weight per day.

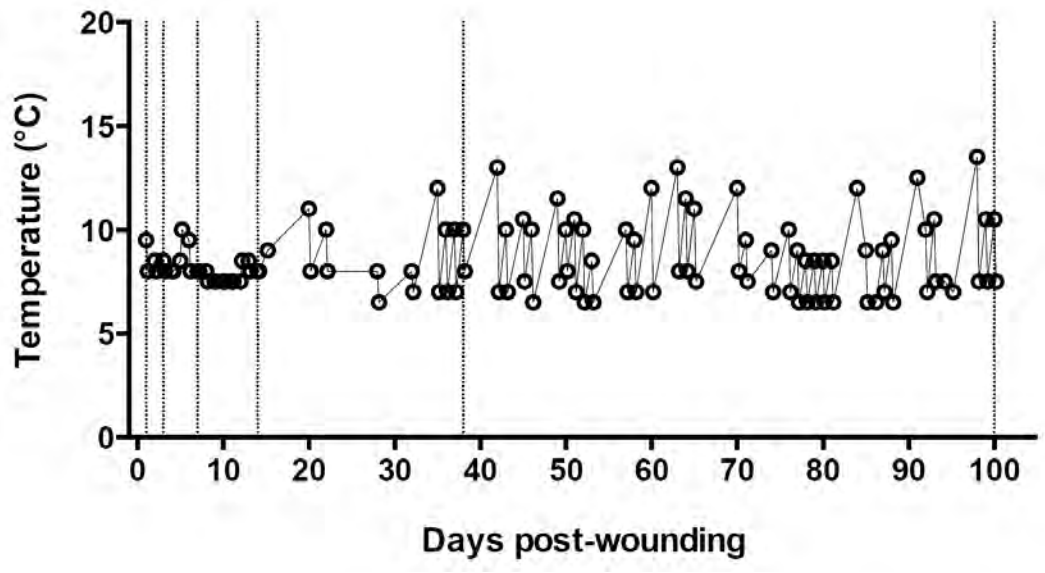


Figure 1. Temperature measurements to nearest 0.5°C during the experiment. Vertical dotted lines represent sampling days.

2.2. MacroGard

MacroGard (Biorigin, Oslo, Norway) is a β-glucan product prepared from Baker's yeast (*Saccharomyces cerevisiae*) cell walls. It contains >60% β-1,3/1,6-glucans. It is thus a source of a

range of potential PAMPs, with β-glucans constituting the majority of these. MacroGard stock solution was prepared by dissolving 0.5 g of MacroGard in 500mL of MilliQ water while stirring for 1h at 90°C. The solution was then autoclaved (121°C, 15min, 1atm).

2.3. Experimental setup

The fish were divided into two identical 200L tanks with aerated tap water. Half of the fish in each tank were wounded and the other half were left unharmed. To one of the tanks was added MacroGard stock solution to a final concentration of 0.1mg/L. The other contained only tap water. Thus the experiment contained four treatment groups of fish. For the first 14 days after the experiment was initiated the tanks were sealed off from the filter system and water was changed daily with tap water and MacroGard added anew. After 14 days the treatment with MacroGard was stopped, the tanks were re-opened to recirculation and the two tanks were now part of the same recirculation system for the remainder of the experiment.

2.4. Wounding

Anaesthesia was induced in ten fish at a time in 125 mg/L tricaine methanesulfonate (MS-222, Sigma-Aldrich, Broendby, Denmark) after which the fish were moved to 50 mg/L MS-222. Four cylinders of tissue were excised with a 6mm biopsy punch (Miltex, York, USA) and scalpel from an area between the lateral line and the dorsal fin on the left side of the fish. The wounds were approximately spaced 1cm apart, they were 3mm deep and penetrated into the muscle. The fish were alternately released into each of the two tanks with or without MacroGard. A mock procedure was carried out with the non-wounded control fish. The procedure was repeated until each experimental tank contained 77 fish (only 60 fish from each tank were used for this experiment).

2.5. Sampling

On day 0 (the day of wounding), five non-wounded/non-treated fish were sampled as baseline controls (figure 2). On days 1, 3, 7, 14, 38 and 100 post-wounding (dpw) samples were collected from five fish from each group (wounded/ MacroGard, non-wounded/MacroGard, wounded/ no MacroGard, non-wounded/no MacroGard,).

Five fish at a time were taken from one of the tanks and killed in 125 mg/L MS-222. Weight and fork length was recorded (figure 3) and an image of the wounded area was acquired using a multispectral imaging device (VideometerLab, Hoersholm, Denmark).

The fish was then immersed in liquid nitrogen until frozen solid after which it was moved to a -20°C freezer until all fish of the given sampling day were killed and frozen. Immediately hereafter, the fish were then removed from the freezer a few at a time. It was not possible to

sample the fish at -20°C so they were kept at room temperature until they had reached a temperature that allowed sampling. The fish were still frozen at this time, which allowed for sampling of the periphery of each wound in an accurate and consistent manner with an 8mm biopsy punch. The frozen skin and muscle separated relatively easily from each other compared to fresh tissue. The muscle tissue was put into cryotubes which were initially placed in liquid nitrogen and then stored at -80°C until RNA extraction. Also a similar area was sampled from the right side of wounded fish (internal control) as well as the left side of non-wounded fish (external control).

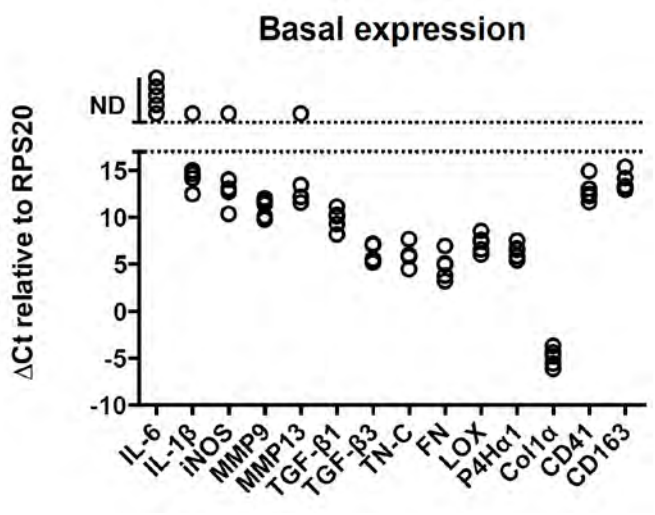


Figure 2. Basal expression of the investigated genes measured on muscle from 5 individual rainbow trout on the day the experiment was initiated, but before wounds were inflicted. Values are plotted as ΔCt values. Open circles above the dotted lines represent samples for which an expression was not detected (ND).

2.6. Extraction of RNA and reverse transcription

RNA was extracted with the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Broendby, Denmark) and following the manufacturer's instructions. Frozen tissue was immersed in lysis buffer and disrupted by sonication (using a Sonics Vibracell sonicator, Sweden, fitted with a model CV 18 probe and set to 40s, pulse 2, amplitude 70 %). The RNA was eluted in a final volume of 30µl. The extracted RNA was treated with DNase 1, Amplification Grade (AMPD1-1KT, Sigma-Aldrich, Broendby, Denmark) according to manufacturer's instructions to remove contaminating genomic DNA. RNA quantity and purity (OD_{260}:OD_{280} ratio) was then measured on a NanoDrop 1000 (Thermo Fisher Scientific, Germany). The sampling procedure was not favorable for the preservation of RNA, and considerable degradation was apparent in some samples. Samples with an OD_{260}:OD_{280} ratio of less than 1.9 and/or an RNA quantity of less than 20 ng/µL were discarded (26 samples out of a total of 180 samples).

The RNA was reverse transcribed with TaqMan® Reverse Transcription reagents (N808-0234, Applied Biosystems, Foster City, USA) primed with random hexamer primers (2µl 10x RT buffer, 4.4 µl 25mM MgCl2, 4µl dNTP mix, 1µl Random Hexamer Primers, 0.4µl RNase inhibitor, 1.25µl Multiscribe Reverse Transcriptase and 400ng RNA diluted in 6.95µl RNase free water; final reaction volume 20µl). Samples were prepared in 0.2µl tubes which were placed in a thermocycler (Veriti, Applied Biosystems, Foster City, USA) and run for 10min at 25°C, 60min at 37°C and finally 5min at 95°C. The samples were then diluted 1:10 in MilliQ water and stored at -20°C.

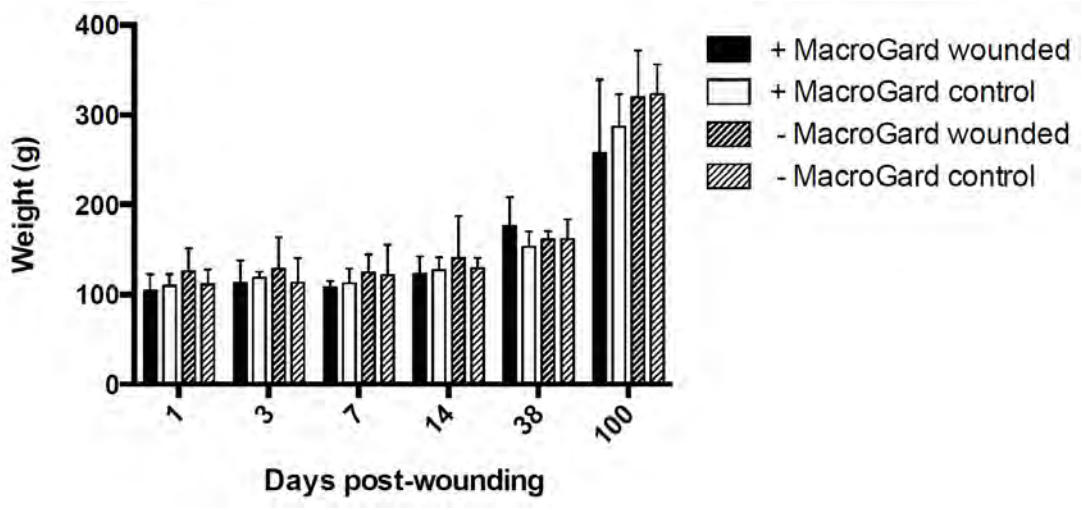


Figure 3. Weight of the sampled fish (shown as mean with standard deviation). There was no significant difference in growth of the experimental groups over the course of the experiment.

2.7. Primer design

When possible already published primer sets were used. When not, these were designed from sequences deposited in GenBank (Table 1). In the cases of genes where relevant annotated rainbow trout nucleotide sequences were not found in the NCBI database, annotated nucleotide sequences of closely related species were used to make BLAST searches in salmonid EST databases. Primer sequences were designed using the Primer3 software (Untergasser et al., 2012). Primers were designed for a 60°C annealing temperature and an amplicon length between 75 and 150bp.

Gene		Primer sequence	Reference	Amplicon length (bp)	Primer efficiency
RPS20	F R P	AGCCGCAACGTCAAGTCT GTCTTGGTGGGCATACGG TGTGCAGACCTTATCCGTGGAGCT	(Ingerslev et al., 2010)	104	1,905
IL-1β	F R	GCTGGAGAGTGCTGTGGAAGAACATATAG CCTGGAGCATCATGGCGTG	(Castro et al., 2011)	179	1,748
IL-6	F R	ACTCCCCTCTGTCACACACC GGCAGACAGGTCCTCCACTA	(Castro et al., 2011)	91	1,870*
iNOS	F R	CCAACAGGTGTCCGTTTTCT TGATGTGCATGGTTGGTTCT	NM_001124359	104	1,894*
CD163	F R	CAACGTCATCCAGCCAGATA ACTGTGGGGGTACAAACCAA	FP321512	78	1,932*
TGF-β1	F R	AGCTCTCGGAAGAAACGACA AGTAGCCAGTGGGTTCATGG	X99303	136	1,874
TGF-β3	F R	TCCACGAGCCTAAGGGATAC CTCAGGGTTCAGGGTGTTGT	AJ318928	116	1,94
MMP9	F R	AGACATGGGAGCCTCTCTGA TGCTTTTCCAAATGACACCA	AJ320533	130	1,759
MMP13	F R	GCTTCACCACCTTCGACAAT ATGGAGTTGTCCACCTCAGC	(Sutherland et al., 2011)	103	1,814
LOX	F R	TGGCACAAGGTACCATCAGA AGCCGAACTTGACAAGCAAT	EF514520	139	1,899
P4Hα1	F R	TGAATCCTTCCTTGGTGTCC CAACGCCTTCAAACTGATGA	DV200837	86	1,993
FN	F R	AACGCCCTGAAGAAACTGAA TGAGGAGGAGGATGTGGTTC	CU071374	119	1,91
TN-C	F R	AAATTCACGATCGCAGAACC AGTGCACAGTTGGTGACAGC	CU073092	149	1,884
Collα1	F R P	TGAGGGAACTCCTGGTAACG ACTCACCACGTTCTCCCTTG CTTCTGGTCGCGATGGTGCT	(Ingerslev et al., 2010)	74	1,8
CD41	F R	AGGAGCATCCTGCTGACCTA CATGGTGATGGGTAGGGAAC	CX257497	123	1,856

Table 2. Primer sequences used for qPCR. F, forward primer, R, reverse primer, P, probe. The asterisks indicate primer pairs for which efficiency could not be calculated due to low expression. Since the RNA quality was suboptimal amplification efficiency was influenced by amplicon length, and efficiency estimates were instead extrapolated based on efficiency of the other primer sets.

2.8. Gene expression analysis

Gene expression was quantified with real-time quantitative polymerase chain reaction (qPCR) using SYBR© Green on a Stratagene MX3000P™ thermocycler for all genes except for collagen type I α-chain. Here the fluorescence in the reaction was instead provided by a dual-labelled TaqMan® probe conjugated with a 5' FAM fluorophor and a 3' BHQ_1 quencher for collagen type I α-chain as well as RPS20 (Sigma-Aldrich, Brøndby, Denmark). We used ribosomal protein S20

(RPS20) as a reference gene, as it was previously shown to be stably expressed in injured and non-injured muscle of salmonids (Ingerslev et al., 2010, Ingerslev et al., 2006). The primer set for RPS20 was efficient for use with or without probe.

For the SYBR© Green qPCR a master mix was prepared from SYBR© Green JumpStart™ Taq ReadyMix™ kit (S4438, Sigma-Aldrich, Broendby, Denmark) using the following relative volumes (shown as final volume per well): 12.5µL SYBR© Green JumpStart™ Taq ReadyMix™, 0.5µL ROX reference dye (deleted 10X) and 9.0µL DEPC-treated water (Sigma-Aldrich, Broendby, Denmark). This mix was aliquoted into tubes corresponding to the number of cDNA samples tested on the given day and diluted cDNA was added (corresponding to 1.0µL per well). The mix was transferred to a 96-well plate and forward and reverse primers were added (1.0µL of each per well). Primer specificity was subsequently confirmed by a melt curve analysis, which was initiated with a denaturing step for 1min at 94°C followed by a gradual increase from 60°C to 94°C with fluorescence measurements at short intervals.

For the probe-based qPCR each well contained 5µL JumpStart™ Taq ReadyMix™ kit (D7440, Sigma-Aldrich, Broendby, Denmark), 0.45µL forward and reverse primer (20µM), 1µL probe (2.5µM) and 2.6µL DEPC-treated water (Sigma-Aldrich, Broendby, Denmark). For probe-based as well as SYBR© Green-based qPCR the cycling conditions were 10min at 95°C followed by 40 cycles of 15s at 95°C and 1min at 60°C.

Amplification efficiency was calculated with triplicate dilution series. However, in the case of iNOS, CD163 and IL-6, expression was so low that reliable dilution series could not be made. Due to the poor RNA quality there was an effect of amplicon length on the PCR reaction efficiency, and iNOS, CD163 and IL-6 efficiency was instead estimated based on measured amplification efficiencies of the other primer sets.

2.10. Statistical analysis of gene expression data

In a situation where there are considerable differences in amplification efficiency between the gene of interest (GOI) and the reference gene (Ref) one would normally use the Pfaffl method (Pfaffl, 2001) to calculate expression differences. However, with this method efficiency is applied to group averages. Due to few replicates in some experimental groups we used regression models, and the Pfaffl approach was thus not applicable. We instead transformed the C_t values for GOI and Ref in the following way:

$$New\ Ct = (Observed\ Ct) \times \log_2(efficiency)$$

$$New\ dCt = New\ Ct_{GOI} - New\ Ct_{Ref}$$

The New dC_t values were then used in the regression analysis.

Some of the investigated genes had a low basal expression, which meant that the cycle threshold was not reached within 40 cycles (the programmed number of cycles in the amplification step) for some samples. These samples were censored. Most of the censored samples were internal (I) or external (E) control samples, whereas most wound (W) samples gave a Ct value. Data for these genes were analyzed with tobit regression (a censored regression model). The tobit regression builds on the assumption that the data have a normal distribution, since this was the case for the genes that were analyzed without censoring.

For each GOI there are two sets of analyses. In the first set of analyses data is used from the wounded side of the fish and the external control fish (data set WE) and the second set of analyses uses data from the non-wounded side of the wounded fish (internal control) and the external control fish (data set IE).

For both data sets the cycle differences are analysed using normal or tobit regression models. To test the effect of MacroGard an initial model is fitted including the main effects of day (1, 3, 7, 14, 38 and 100), type of fish (E or W/I) and MacroGard (yes/no) and the pairwise interactions between these factors. The initial model is reduced by stepwise testing using likelihood ratio tests. Results are considered significant at $P<0.05$. Model control is carried out using regression plots and QQ plots to verify the assumptions underlying the normal models.

The genes for which some samples were censored were MMP13 (1), IL-1β (33), IL-6 (99), iNOS (29), CD41 (3) and CD163 (46) (the number of censored samples out of the total 154 are shown in brackets). IL-6 expression was very low in homeostatic tissue and very few control samples (internal as well as external) reached the threshold within 40 cycles. All external control samples were censored on 7dpw, 38dpw and 100dpw. Thus we could not calculate estimates for all combinations of variables, and we could not plot estimated $\Delta\Delta C_t$ values as for the other genes. Instead we show the IL-6 results as observed ΔC_t values (figure 5).

2.9. Image acquisition and analysis

Multispectral images of the wounds were acquired using a VideometerLab (Videometer A/S, Hørsholm, Denmark). The VideometerLab produces standardized images with diffuse illumination from light-emitting diodes. The wound edge was outlined manually and the size of the open wound area was determined using MatLab (The MathWorks Inc., Natick, MA, USA). Differences in open wound area between MacroGard-treated and control groups were tested using an unpaired t-test. Wound size relative to original size (%) is plotted as mean with standard deviation (figure 4).

3. Results

3.1. Effect of MacroGard treatment

There was no effect of MacroGard on the visual wound closure kinetics (figure 4). On the gene expression level the effect of MacroGard treatment was weak. There was no effect on any of the non-censored genes, but some effect on the expression of MMP13 14dpw (5.4-fold lower expression in the MacroGard-treated WE group, P=0.0002), IL-6 14dpw (8.6-fold higher expression in the MacroGard-treated WE group, P=0.0062) and iNOS 1dpw (33.6-fold lower expression in the MacroGard-treated IE group, P=0.0043; 8.4-fold lower expression in the MacroGard-treated WE group, P=0.0072) and 100dpw (29.7-fold lower expression in the MacroGard-treated IE group, P=0.0041; 13.8-fold lower expression in the MacroGard-treated WE group, P=0.0015) (not shown).

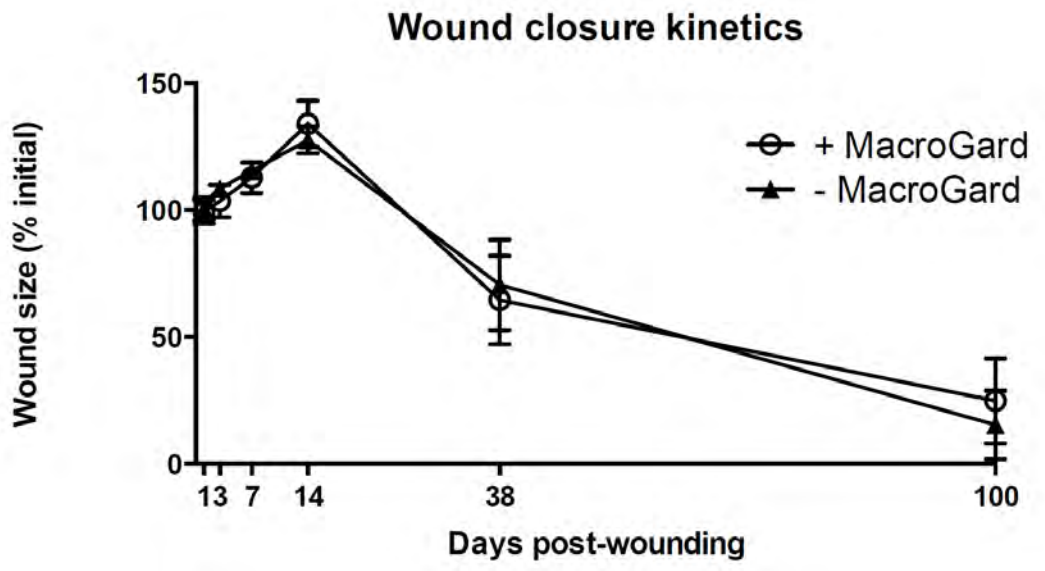


Figure 4. Wound closure kinetics shown as relative to original wound size at time of infliction (%). Note Log2 scale of X-axis. There is an initial increase in wound size until day 14 post-wounding after which wound size decreases. There was no significant effect of MacroGard treatment on wound closure.

3.2. Systemic effect of wounding

There was a minor gene expression effect of wounding in non-wounded internal control tissue compared to non-wounded external control tissue. The greatest effect was found at 14dpw. On this day MMP9 (4.9-fold, P=0.0007), MMP13 (6.4-fold, P=0.0008), TGF-β1 (2.5-fold, P=0.0189), LOX (2.8-fold, P=0.0049) and P4Hα1 (2.2-fold, P=0.0137) were expressed at significantly higher

levels in internal control tissue than in external control fish. At 1dpw iNOS (17.9-fold, P=0.0187) was expressed at significantly lower levels in internal control tissue than in external control fish adjusted for MacroGard treatment.

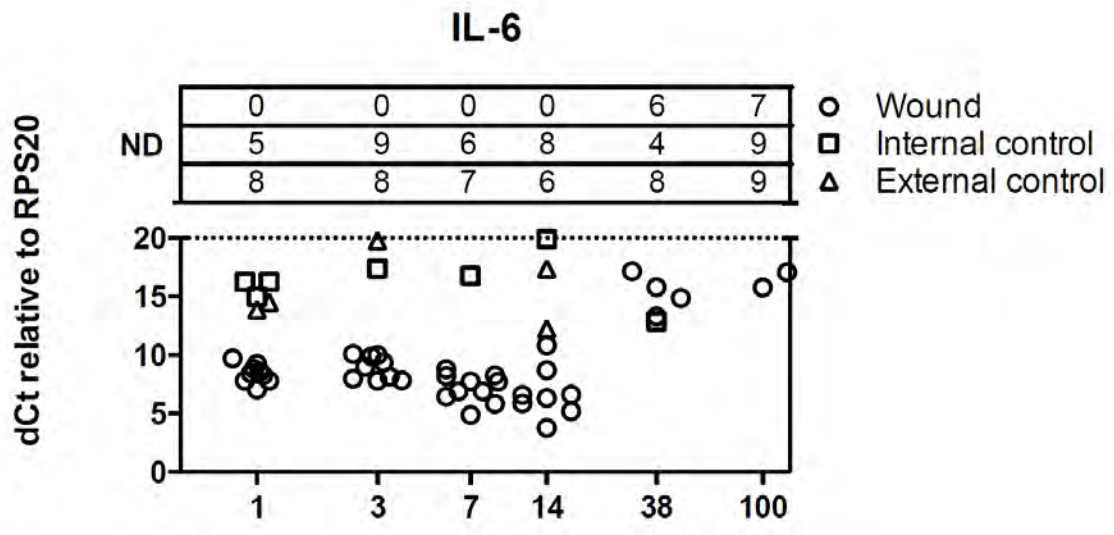


Figure 5. Expression of IL-6 was very low in control muscle, and in most cases beyond the level of detection. The table (top) shows the number of individual fish for which no IL-6 expression was detected (ND). The two MacroGard treatment groups have been pooled, and only the sample groups wounded (top), internal control (middle) and external control (bottom) remain. Individual ΔCt values are shown in the scatter plot below. Each group contain a maximum of 10 fish.

3.3. Effect of wounding on gene transcription in the wound area

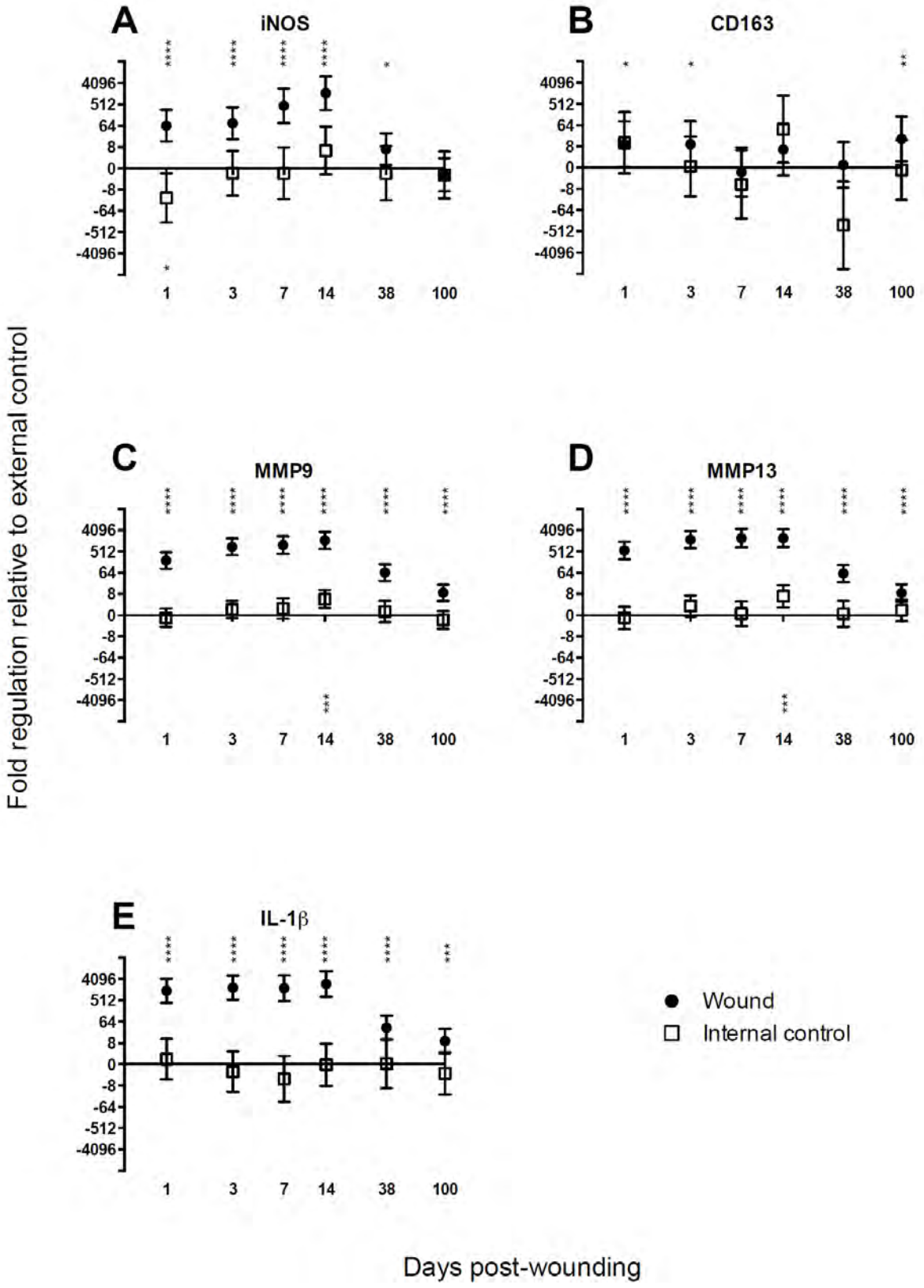
The expression pattern of the investigated genes can largely be divided into two groups: Genes with an early induction until 14dpw; and genes with a late induction after 14dpw (figure 6).

3.3.1. Genes induced during the inflammation phase

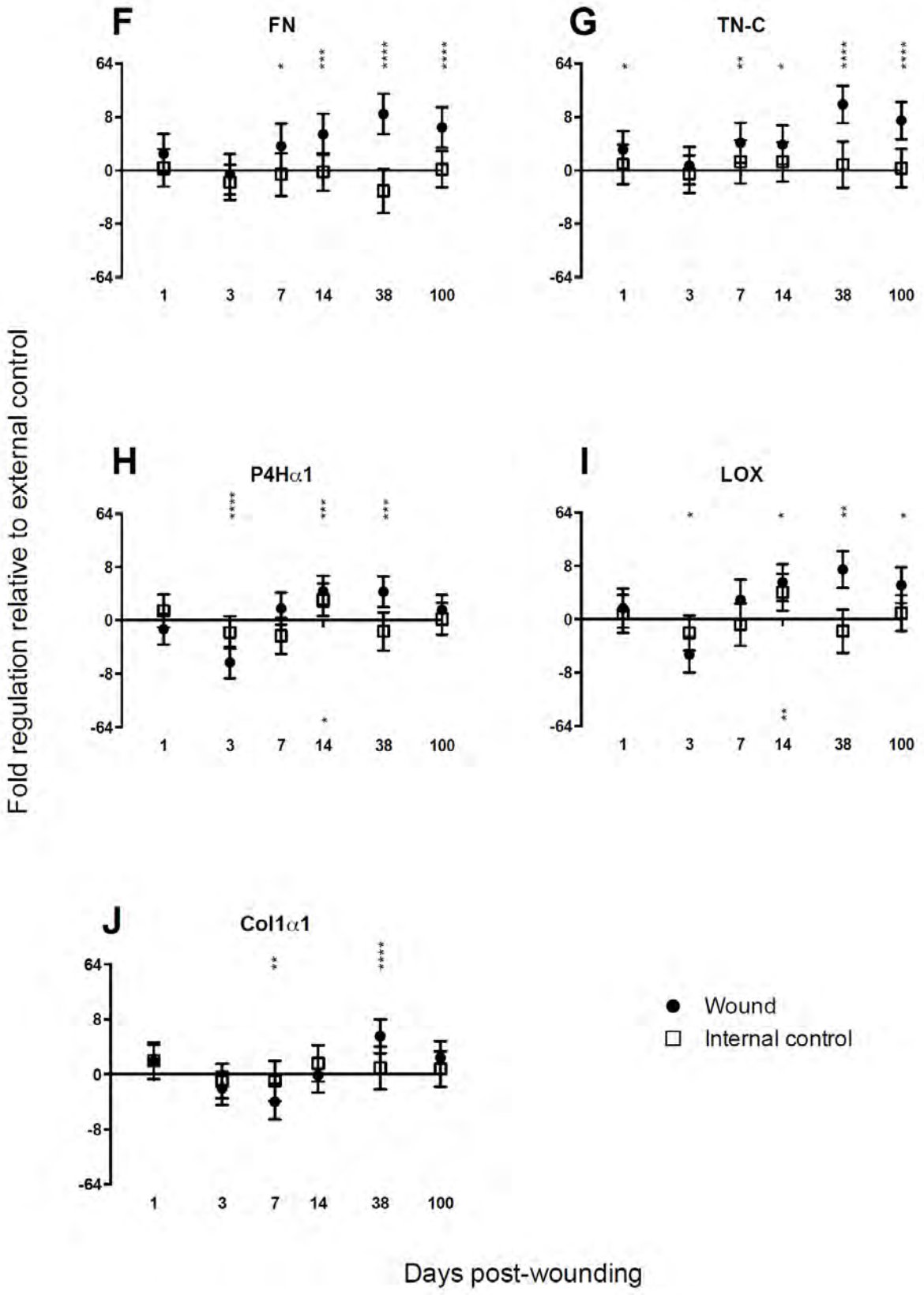
IL-1β is highly upregulated 1dpw through 14dpw (1288-fold 1dpw; 1734-fold 3dpw; 1642-fold 7dpw; 2511-fold 14dpw, all days P<0.0001). Between days 14 and 38 there is a sharp drop in IL-1β transcription, but it is still significantly higher than external control fish (35-fold 38dpw, P<0.0001; 9.6-fold 100dpw, P=0.0002) (figure 6E).

MMP9 and MMP13 both follow an expression pattern very similar to that of IL-1β. The expression of these MMPs is, however, not upregulated as fast IL-1β and peaks at a lower level. The upregulation is highly significant on all days for both genes (P<0.0001).

MMP9 is upregulated: 211-fold 1dpw; 810-fold 3dpw; 959-fold 7dpw; 1507-fold 14dpw; 65-fold 38dpw; 9.1-fold 100dpw (figure 6C). MMP13 is upregulated: 571-fold 1dpw; 1631-fold 3dpw; 1896-fold 7dpw; 1895-fold 14dpw; 65-fold 38dpw; 9.1-fold 100dpw (figure 6D).


A
iNOS
B
CD163
C
MMP9
D
MMP13
E
IL-1β
4096
512
64
8
0
-8
-64
-512
-4096
1
3
7
14
38
100
Fold regulation relative to external control
Days post-wounding
Wound
Internal control

Continued…


F
FN
G
TN-C
H
P4Hα1
I
LOX
J
Col1α1
64
8
0
-8
-64
1
3
7
14
38
100
Fold regulation relative to external control
Days post-wounding
Wound
Internal control

Continued…

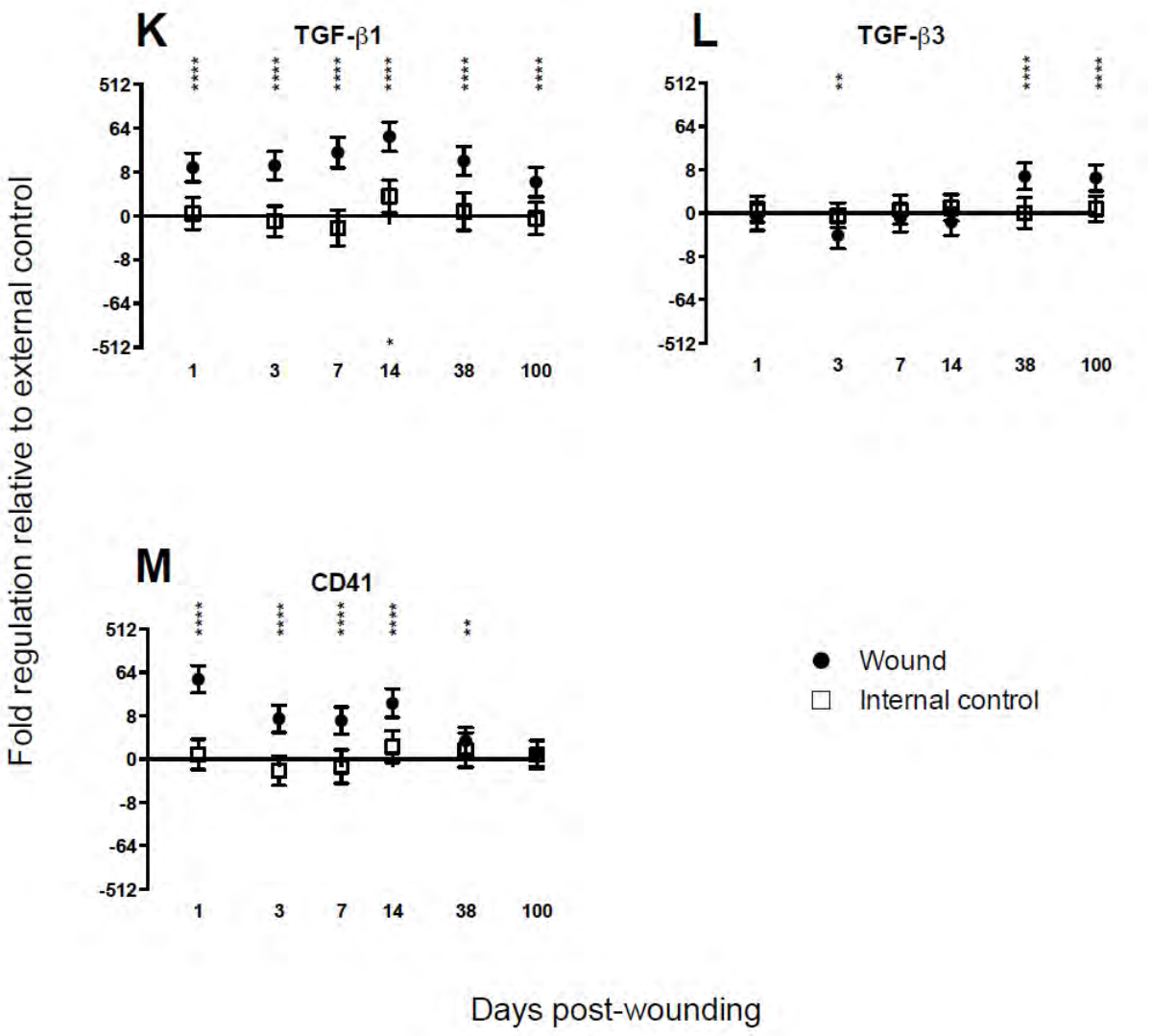


Figure 6. Expression of the investigated genes shown as fold difference regulation in wound tissue (closed circles) and internal control tissue (open squares) relative to tissue from external control fish. Both axes are log2. Asterisks above the X-axis represent significant differences between wound and external control and asterisks below the X-axis represent differences between internal and external controls. *0.05>P>0.01, **0.01>P>0.001, ***0.001>P>0.0001, ****0.0001>P.

iNOS expression in wounds relative to external control tissue is upregulated 1dpw through 38dpw with a peak at 14dpw (65-fold 1dpw; 83-fold 3dpw; 449-fold 7dpw; 1532-fold 14dpw, all days 1-14 $P<0.0001$; 6.4-fold 38dpw, $P=0.0184$). At 100dpw expression is similar to that of control tissue (figure 6A).

It was not possible to test for wound-induced differences in IL-6 expression with or without censoring as very few control samples gave a C_t value. However, from figure 5 it is evident that IL-6 expression was highly upregulated in wounds through 14dpw.

CD41 expression is significantly upregulated 1dpw through 38dpw with the highest peak 1dpw and a smaller peak 14dpw (46-fold 1dpw; 7.0-fold 3dpw; 6.3-fold 7dpw; 15-fold 14dpw,

all days 1-14 P<0.0001; 2.4-fold 38dpw, P=0.0065). At 100dpw expression is similar to that of control tissue (figure 6M).

TGF-β1 expression is upregulated with high significance (P<0.0001) on all days. Expression levels peak at 14dpw (9.8-fold 1dpw; 10.8-fold 3dpw; 20-fold 7dpw; 43-fold 14dpw; 13-fold 38dpw; 4.9-fold 100dpw) (figure 6K).

3.3.2. Genes induced after the inflammation phase

These remaining investigated genes exhibit a much more limited regulation that rarely exceeds one order of magnitude.

TGF-β3 is downregulated 3dpw (3.0-fold, P=0.0011) and upregulated 38dpw and 100dpw (5.4- and 5.8-fold respectively, P<0.0001) (figure 6L).

CD163 expression varied considerably between individual fish and although CD163 expression was upregulated 10-fold 1dpw and 3dpw, this was only borderline significant (P=0.0432 and P=0.0458, respectively). The main difference in CD163 expression between wound and external control was at 100dpw (17-fold upregulation, P=0.0057) (figure 6B).

TN-C and FN are upregulated 7-100dpw with a peak at 38dpw. TN-C is upregulated: 2.9-fold 7dpw, P=0.0078; 2.7-fold 14dpw, P=0.0101; 13-fold 38dpw, P<0.0001; 6.9-fold 100dpw, P=0.0001. FN is upregulated: 2.6-fold 7dpw, P=0.0370; 4.0-fold 14dpw, P=0.0009; 9.0-fold 38dpw, P<0.0001; 5.3-fold 100dpw, P=0.0001. TN-C is additionally weakly significantly upregulated 1dpw (2.2-fold, P=0.0322)(figures 6F and 6G).

LOX and P4Hα1 are both downregulated 3dpw (1.7-fold, P=0.0185; 4.0-fold, P<0.0001, respectively) and upregulated 14dpw (4.2-fold, P=0.0180; 3.1-fold, P=0.0003, respectively) and 38dpw (6.9-fold, P=0.0015; 3.0-fold, P=0.0004, respectively). LOX is still significantly upregulated 100dpw (3.8-fold, P=0.0237), whereas P4Hα1 expression is at control values (figures 6H and 6I).

ColIα1 expression kinetics is similar to its cognate cross-linking enzymes, but is delayed relative to these with significant downregulation 7dpw (2.8-fold, P=0.0030) and upregulation 38dpw (4.0-fold, P<0.0001)(figure 6J).

3.4. Wound closure

There was no effect of MacroGard-treatment on the wound closure kinetics. The open wound area initially enlarged until 14dpw, after which it became progressively smaller on the following days (figure 7). The wounds closed with relatively little contraction and had only completely closed (or very nearly so) in two of the fish 100dpw.

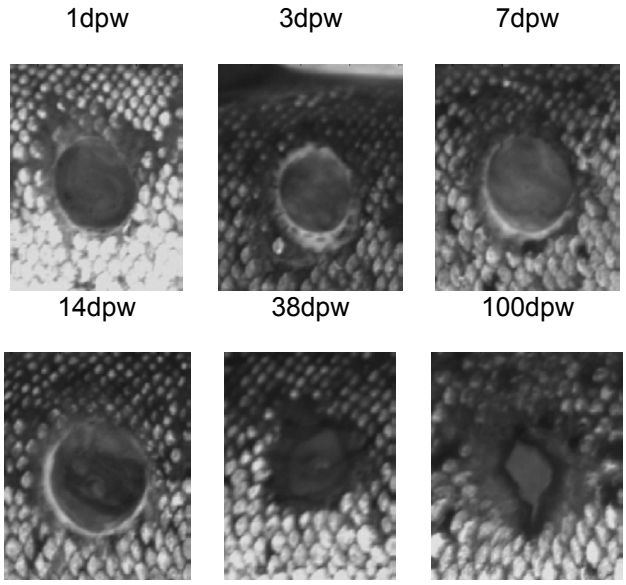


Figure 7. Grayscale VideometerLab images of wounds taken on different days after wounding (dpw). The six images are from six different fish.

4. Discussion

4.1. *PAMP bathing did not affect wound healing*

Wound closure was not affected by the added PAMPs and only the genes MMP13, IL-6 and iNOS were transcribed at different levels in wounds of MacroGard-treated and non-treated fish. These genes are all highly transcribed in M1 polarized macrophages (Castillo-Briceño et al., 2010, Costa et al., 2011, Kou and Babensee, 2011, Rigamonti et al., 2013), which is the cell type most often associated with recognition and response to PAMPs (Boltaña et al., 2011). However, compared to the effect of wounding on the regulation of transcription of these genes (more than three orders of magnitude) the effect of MacroGard was minor. The only significant effect of MacroGard on MMP13 and IL-6 expression was at 14dpw. When injecting rainbow trout intraperitoneally with β-glucans Jørgensen et al (Jørgensen et al., 1993) also found the largest effect on bactericidal activity of isolated head kidney macrophages two weeks after injection. The limited response to PAMP stimulation in rainbow trout is probably not an effect of the low temperature since rainbow trout macrophage activation is not inhibited at low temperatures (Hardie et al., 1994). However, stress from temperature fluctuations could be part of the explanation, since heat-shock protein 70 released as a result of temperature stress has anti-inflammatory properties (Borges et al., 2012, Deane and Woo, 2011, Harper and Wolf, 2009). Yet another explanation for the minor effect of PAMP bathing on gene expression in muscle is the fast epithelial coverage of wound in fish (Rai et al., 2012, Ream et al., 2003). Thus the wounded muscle is not exposed to the external environment for a very long time. For wounded common carp subjected to a similar treatment as in the present study we recently reported no observed effect on gene expression, but a faster contraction of the wounds (Przybylska-Diaz et al., 2013). The PAMP bathing thus

probably mainly affects the epidermis, which could be speculated to convey signals to fibroblasts to induce differentiation into contractile myofibroblasts. From mammals it is known that skin structure affects wound contraction (Volk and Bohling, 2013), and differences in skin structure may explain the different wound contraction responses to PAMP stimulation in the two species. Even in the absence of PAMP stimulation the extent of contraction of wounds in carp is greater than in rainbow trout (personal observations). Another possibility is that carp are highly responsive to PAMP stimulation compared to rainbow trout, since they naturally inhabit waters which are more turbid and rich in microorganisms. It is thus important with a fast response to PAMPs if the epithelial barrier is breached. In fact, carp are known to tolerate higher bacterial pathogen loads without developing signs of disease and isolated carp phagocytes have a stronger response to bacterial pathogens than those of rainbow trout (Sakai et al., 1996).

4.2. *Gene expression in the wound*

Gene expression during healing of injured muscle has not previously been studied over such a prolonged period in a teleost. The results show that phases, which last for days in mammals or warm-water cyprinids last for weeks or even months in this cold-water teleost.

4.2.1. Hemostasis and thrombocytes

Hemostasis is one if the first events that take place following tissue damage in vertebrates. Hemostasis involves vasoconstriction, activation of the blood clotting cascade, and adhesion and activation of platelets or thrombocytes. Thrombocytes are nucleated lower vertebrate equivalents of platelets. Their involvement in hemostasis is conserved from fish to mammals (Lang et al., 2010), and the integrin α-chain CD41 is specifically expressed by platelets/thrombocytes at high levels and by very early hematopoietic progenitors at low levels in zebrafish as well as humans (Lin et al., 2005). We thus chose CD41 as a marker also for rainbow trout thrombocytes.

Apart from hemostasis, piscine thrombocytes may perform different functions than platelets, since it was demonstrated that rainbow trout thrombocytes are phagocytic (Hill and Rowley, 1998) and possibly antigen-presenting (Köllner et al., 2004). Thus, we speculated that they could persist in wounds for longer than their mammalian counterparts, as well as to actively migrate out of the wounds to sites of antigen presentation. Our results show an early increase in CD41 transcripts at the wound site 46-fold that of control tissue levels 1dpw. The transcript levels are lower from 3dpw onwards, but still significantly upregulated through 38dpw. This expression pattern is consistent with persisting thrombocytes in the wound. It would be tempting to explain the biphasic expression profile by an initial upregulation due to trapping of

thrombocytes and the second (smaller) peak by granulation tissue formation, which is rich in blood vessels and thus in circulating thrombocytes. However, even as late as 100dpw the muscle samples were much more red compared to the pale control samples (and thus more vascularized) and the expression of FN and TN-C (which are important granulation tissue ECM molecules) was still elevated. Degree of vascularization thus seemed to have little effect on the measured expression of CD41. It will be interesting to take a more detailed look at the long-term involvement of piscine thrombocytes in wound healing.

4.2.2. Inflammation

IL-1β was included as a marker for the inflammatory phase, as it is a prototypic marker of inflammation. Whether it is mainly activated by caspase dependent or independent mechanisms is still not entirely clear, but could be species specific (Angosto et al., 2012, Secombes et al., 2011, Vojtech et al., 2012). Among many effects IL-1β attracts macrophages which themselves produce IL-1β and contribute to the inflammatory response (Rider et al., 2011).

We recently showed that when the same wound model was applied to carp IL-1β expression was only significantly upregulated 1dpw and was not significantly different from control values already 3dpw (Przybylska-Diaz et al., 2013). However, in the present experiment inflammation was not resolved until sometime between 14dpw and 38dpw – and even 38dpw and 100dpw IL-1β expression was still significantly elevated, but at much lower levels.

In mammals, inducible nitric oxide synthase (iNOS) is a marker for inflammatory M1 polarized macrophages, and the scavenger receptor CD163 as a marker for M2 polarized macrophages of the M2c subset. iNOS is also considered a marker of classically activated M1 macrophages in fish (Forlenza et al., 2011), while the suitability of CD163 as an M2 marker has not yet been investigated in fish. iNOS can also be expressed by lymphocytes, neutrophils, keratinocytes and fibroblasts after wounding (Bernatchez et al., 2013), but it was recently described that in mice iNOS expression in injured muscle is restricted to infiltrating macrophages and furthermore that iNOS is important for muscle regeneration (Rigamonti et al., 2013).

M2 macrophages are more diverse than the classically activated, inflammatory M1 phenotype. Macrophage polarization is very plastic and a clear-cut distinction between subsets is difficult to establish, but three subsets of M2 are usually described in mammals. These include M2a, -b and -c. CD163 is not expressed by M1 polarized macrophages, but by non-activated circulating monocytes and all three subsets of M2 macrophages, although mostly by the M2c subset (David and Kroner, 2011, Zizzo and Cohen, 2013).

All three subsets potentially play a role in wound healing. The M2c subset has important functions in resolution of inflammation and in regaining tissue homeostasis (Kharraz et al., 2013, Zizzo and Cohen, 2013) and $CD163^{+}$ macrophages are associated with regenerating myofibers in the rat (Arnold et al., 2007).

Markers for M2 macrophages in fish are scarce, and may differ between species. Arginase activity may for the moment be the best marker, but probably mainly for macrophages more closely resembling the M2a subset (Forlenza et al., 2011). In this experiment CD163 was included as a possible novel M2 subset marker in the rainbow trout. The expression of CD163 was generally quite low and variable in our experiment, and the observed regulation of transcription was thus not highly significant. However, there was an early upregulation of CD163 in the wound 1dpw and 3dpw, which may reflect an influx of non-activated monocytes expressing CD163. These then assumedly switch to an M1 phenotype from 7dpw to 38dpw when CD163 expression in the wound is not significantly different from control tissue. At 100dpw CD163 is upregulated, and may mark the switch to an anti-inflammatory and tissue homeostatic milieu.

IL-6 is a pleiotropic cytokine predominantly expressed during the inflammatory phase, and is involved in several events in wound healing, such as promotion of neutrophil apoptosis (Ganeshan et al., 2013) and re-epithelialization, angiogenesis and collagen accumulation (Lin et al., 2003, O'Reilly et al., 2012). IL-6 is also involved in muscle regeneration through differentiation of myogenic progenitors into mature myotubes (Heredia et al., 2013). IL-1β and PAMPs induce IL-6 expression in rainbow trout macrophages (Costa et al., 2011), and in our experiments IL-6 expression was higher in MacroGard treated fish 14dpw. IL-6 expression is strongly upregulated in wounds only until 14dpw, which indicates a limited involvement of this cytokine (or myokine, as it has also been called (Pedersen and Fischer, 2007)) in trout muscle regeneration, and perhaps a limited regeneration capacity of trout muscle altogether.

The matrix metalloproteinases MMP9 and MMP13 degrade mainly basement membrane collagens and fibrillar collagens respectively (Leeman et al., 2002, Van den Steen et al., 2002). They are both important for several early events during wound healing, including epithelial migration, angiogenesis, granulation tissue formation and wound contraction (Hattori et al., 2009, Toriseva et al., 2012). They also contribute to inflammation as MMP13 induces MMP9 expression (Toriseva et al., 2012) and MMP9 can boost inflammation by activation of proinflammatory cytokines (Van den Steen et al., 2002). The expression of these proteases to a large extent mirrors that of IL-1β, and they thus are involved in wound healing mainly during the inflammatory stage.

4.2.3. Production and remodeling of ECM

In mammals as well as fish there are three isoforms of TGF-β: 1, 2 and 3. However, a fourth isoform has been discovered in gilthead seabream (*Sparus aurata*) and a second TGF-β1 paralogue in rainbow trout (Funkenstein et al., 2010, Maehr et al., 2013). TGF-β1 is a profibrotic cytokine (Haddad et al., 2008, Wick et al., 2013), which is required for differentiation of fibroblasts into myofibroblasts in the presence of a stiff matrix and the ED-A splice form of fibronectin (Hinz, 2010), and thus for extracellular matrix production and wound contraction. Conversely, the isoform TGF-β3 is often associated with regeneration and ameliorates the profibrotic effects of TGF-β1 (Klass et al., 2009, Turner and Badylak, 2012). Fetal wounds heal with perfect regeneration, and here TGF-β3 expression is increased and prolonged compared to adult wounds (Klass et al., 2009, Rolfe and Grobbelaar, 2012, Shah et al., 1995). TGF-β3 is also expressed for longer than the two other isoforms in the regenerating zebrafish fin (Page et al., 2013). The publication by Page et al (2013) is the first on TGF-β3 expression fish, and the present is the first from rainbow trout. In our experiments the basal expression level of TGF-β3 is greater than that of TGF-β1, but TGF-β1 is more induced throughout the investigated 100 days. TGF-β3 is not upregulated until quite late in wound healing, and this is preceded by induction of ECM-related genes. Considering the extended upregulation during the zebrafish fin, which represents a structure that heals with almost perfect regeneration, the upregulation of TGF-β3 expression in rainbow trout muscle could thus be too little too late to prevent fibrosis and support regeneration.

The ECM glycoproteins FN and TN-C are both laid down as a replacement for the initial fibrin clot to form the granulation tissue, which is rich in blood vessels (Satish and Kathju, 2010). Later these are replaced by collagen type I (Lorenz and Longaker, 2003). Collagen type I is the main fibrillar collagen found in muscles (Myllyharju, 2008). We included FN and TN-C as markers of early granulation tissue and collagen type I α1-chain (ColIα1) as a marker for more mature tissue. In addition, we included prolyl 4-hydroxylase α1-chain (P4Hα1) and lysyl oxidase (LOX) as markers of the mature tissue production and remodeling phases. These are enzymes responsible for collagen triple helix formation and cross-linking, respectively (Franklin and Hitchen, 1989, Myllyharju, 2008, Xiao and Ge, 2012). They are thus important for the functional and textural properties of muscle. It is interesting that the upregulation of the enzymes responsible for processing of single collagen chains to provide tissue structure precedes the upregulation of ColIα1. However, collagen production is still maintained at high levels (figure 2), and the elevated expression of LOX and P4Hα1 indicates fibrosis in the healing wound. This is

supported by textural analyses showing that injured rainbow trout muscle tissue is tougher one year post-wounding than muscle of non-wounded fish (data not shown).

4.3. Gene expression in non-injured muscle was only slightly influenced by wounding elsewhere

The difference between gene expression in internal and external control samples was very limited. Inflammation in the wound peaked at 14dpw and on this day expression of the enzymes MMP9, MMP13, LOX and P4Hα1 as well as the growth factor TGF-β1 was significantly higher. This indicates a mild fibrotic effect of wounding on non-injured muscle systemically.

There is still a lot to learn about fish wound healing, and it would be interesting to investigate further what role factors such as phylogeny, temperature and swimming activity has on the progression of wound healing and on the effect of PAMPs. Here we reported greatly prolonged healing dynamics of rainbow trout deep cutaneous wounds. Wound closure, Inflammation, granulation tissue formation lasted for weeks instead of days as observed in most other vertebrates. CD163 expression was consistent with use as a marker of M2 polarized macrophages, although this requires further investigations to establish firmly. CD41 expression indicates that active thrombocytes are retained at wound site for several weeks.

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